THE REPRODUCTIVE HEALTH OF WOMEN TREATED FOR CANCER IN CHILDHOOD

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Abstract

The survival rates for childhood cancer have dramatically increased since the 1960s and at the start of the 21st century, more than 1 in 1000 young adults in their third decade was a survivor of childhood cancer. The aim is to achieve a cure with minimal late effects from the treatment, with the reproductive axis being vulnerable to both radiotherapy and gonadal toxic chemotherapy. This thesis addresses aspects of hypothalamic, pituitary, ovarian and uterine function in post pubertal women following treatment for cancer in childhood.

The effect of low dose cranial irradiation (18-24 Gray) on gonadal function was evaluated in long-term survivors of childhood leukaemia. Tracking of urine luteinising hormone (LH), oestrone and pregnandiol demonstrated reduced LH secretion throughout the cycle and particularly during the LH surge, short luteal phases and decreased oestrone production. These data indicate that treatment for childhood leukaemia results in a subtle ovulatory disorder in some patients, probably related to cranial irradiation.

Women treated for childhood cancer, who have progressed spontaneously through puberty and have regular menstrual cycles, may still be at risk of an early menopause. Ovarian reserve was assessed in women with regular menstrual cycles and women with a history of regular cycles who were using the oral contraceptive pill (OCP), for contraception. They were investigated before and 24 hours after an

injection of follicle stimulating hormone (FSH). Women with regular cycles had significantly higher basal FSH, and lower anti-Mullerian hormone levels, and reduced ovarian volume. Women on the OCP had a reduced inhibin B response to FSH and lower antral follicle counts. Therefore, both groups showed hormonal and biophysical evidence of partial loss of ovarian reserve.

Radiotherapy to the abdomen carries a high risk of ovarian failure. The effect on the uterus is less well documented. Ovarian and uterine function were evaluated in women who had received total body irradiation in childhood (14.4 Gray). In women with ovarian failure, uterine function was evaluated before and after 3 months of physiological sex steroid replacement (pSSR). At baseline, uterine artery blood flow and thickening of the endometrium were not detectable. After 3 months of pSSR neither blood flow or endometrial thickness were different from controls. Uterine volume remained smaller, and there was a correlation with age at irradiation. Endometrial samples were obtained and the histology and histochemistry of the endometrium were normal compared with controls. Hormone replacement therapy that achieves physiological sex steroid concentrations improves uterine size, blood flow and endometrial development.

For those young women that have ovarian failure there is no good evidence as to the optimal method of pubertal induction and subsequent cyclical hormone replacement therapy. UK practice was evaluated by postal questionnaire sent to all British Endocrinologists who were members of the European Society for Paediatric Endocrinology. Therapy for pubertal induction was consistent but there was no

consensus on the choice of cyclical hormone replacement. Clinicians' choice was influenced by convenience and acceptability for the patient.

A case is reported of a girl who had clinical and biochemical evidence of ovarian failure after treatment with pelvic radiotherapy and chemotherapy. Prior to treatment she had had ovarian cortical tissue stored and consideration was given to re-implantation. She had a spontaneous conception, and delivered a healthy baby at term. The case illustrates the difficulties in evaluation of ovarian failure, choice of HRT and the advice that should be given to young girls regarding fertility.

This work further elucidates the late effects on reproductive health for women treated for childhood cancer. Uncertainties remain however, regarding the best way to manage these important health issues, both during treatment and in adult life.

Declaration

I declare that this thesis has been composed by myself.

This research was initiated from an idea of Dr Hamish Wallace. I developed the idea and expanded the work into further studies. I performed the literature review, wrote the study proposals, obtained ethical permission, recruited the patients, performed the research, apart from the imaging, endometrial biopsies and the assays, and performed the data analysis with the help of Dr Richard Anderson.

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Dedication

This thesis is dedicated to David Webb, whose encouragement and enthusiasm inspired me to obtain my MD.

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Publications based on thesis

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Abbreviations

AFC	antral follicle count
ALL	acute lymphoblastic leukaemia
AMH	anti Müllerian hormone
AML	acute myeloid leukaemia
Anov	anovulatory
BMI	body mass index
BMT	bone marrow transplant
Chemo	chemotherapy
CML	chronic myeloid leukaemia
ChlVPP	chlorambucil, vinblastine, procarbazine, prednisilone
COCP	combined oral contraceptive pill
CRT	cranial radiotherapy
E2	oestradiol
ER	oestrogen receptors
E1C	oestrone conjugates
FSH	follicle stimulating hormone
GnRH	gonadotrophin releasing hormone
GH	growth hormone
Gy	Gray
HRT	hormone replacement therapy
IGF1	insulin like growth factor 1
IGFBP3	insulin like binding protein 3

IU	international units
LD_{50}	lethal dose 50%
LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
NHL	non-Hodgkin's Lymphoma
P3G	pregnanediol-3-glucuronide
P4	progesterone
PI	pulsatility index
pSSR	physiological sex steroid replacement
PgR	progesterone receptors
RT	radiotherapy
SSR	sex steroid replacement
TBI	total body irradiation
μg	micrograms
UK	United Kingdom
USS	ultrasound scan

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respectively. * $p<0.05$; ** $p<0.002$ vs control cycles; † $p<0.05$ short vs normal ALL cycles. (b) Daily LH excretion centred on the day of onset of the LH surge (cycle day 0) in control cycles (filled circles, n=16) and cycles or ALL subjects with normal (open triangles, n=24) and short (n=15) luteal lengths. Mean ± sem.

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Urinary steroid excretion in control and ALL subject cycles. (a) Early (days 2-5) and (b) late (days 6-12) follicular phase E1G excretion; (c) luteal phase E1G excretion; (d) luteal phase P3G excretion. Controls (open bars, n=16), ALL subjects (filled bars, n=39). The stippled and hatched bars represent LH excretion in the subgroups of ALL patient cycles with short (n=15) and normal (n=24) luteal phase length respectively. ** $p<0.01$ vs control; † $p<0.05$ vs short luteal length ALL cycles.

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Early follicular phase serum hormones in control and ALL subjects. Controls, open bars, n=16; ALL subjects, filled bars, n=12. * $p<0.05$ vs control. Mean ± sem.

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Serum FSH, AMH, inhibin A, B and proα-C and oestradiol in women with spontaneous menstrual cycles. Controls (open bars, n=11) and survivors of

CHAPTER 1: Introduction

1.1 Background

One in 600 children will develop cancer in the first 15 years of life. Unlike the majority of adult cancers, most paediatric cancers are curable using multi-agent chemotherapy in combination with surgery and radiotherapy. The incidence of childhood cancer is 110 to 130 per million children per year and the relative frequencies of paediatric cancers are shown in Figure 1.1. Over the last three decades there has been a sustained improvement in survival for most forms of childhood cancer. In the 1960s, ALL, the commonest childhood malignancy, had a five-year survival rate of less than 10%. Today, 70% of these children may now be cured (Hann et al., 2000). Following the demonstration in the 1950s that actinomycin was an effective agent in the treatment of Wilms' tumour, there has been steady progress in the development of multi-agent chemotherapy regimens for the majority of childhood haematological and solid tumours. Radiotherapy is highly effective in the treatment of many malignancies but the increasing recognition of the morbidity for children from the late effects of radiation exposure has limited its therapeutic benefit.

At the start of the 21st century, more than 1 in 1000 young adults in their third decade was a survivor of childhood cancer and the number of long term survivors continues to increase. The major challenge for this generation of children's cancer specialists is to sustain the significant improvement in survival rates while at the same time minimizing the treatment-induced late effects. The risk of late effects is directly related to the treatment received. The anticipation of late effects and their detection is

important as they may be amenable to prevention or treatment (Hawkins and Smith, 1996).

The reproductive system is an important site of late effects of anti-cancer treatment (Ogilvey-Stuart et al., 1993). Natural pubertal progression, fertility and successful pregnancy outcome depend on normal hypothalamic, pituitary, ovarian and uterine function. Potential adverse effects on reproductive function in the female may be mediated through the hypothalamo-pituitary-ovarian axis (Littley et al., 1989), the ovary (Wallace et al., 1989) or the uterus (Critchley et al., 1992). One of the more commonly recognised adverse effects of anti-cancer treatments is ovarian failure as a result of depletion of the numbers of primordial follicles leading to a premature menopause (Wallace et al., 2003). However, the late effects of the treatment on reproductive function are difficult to predict with certainty.

1.2 Late effects of treatment on ovarian function

1.2.1 Introduction

There is a relationship between age of menopause in childhood cancer survivors and treatment to which they have been exposed. A review of 1067 women who were more than 5 years out from treatment for cancer, diagnosed during the teenage years, demonstrated a risk of menopause four times greater than controls during their early twenties. Significantly increased relative risks of menopause occurred after treatment with either radiotherapy alone (relative risk 3.7) or alkylating agents alone (relative risk 9.2). Those at greatest risk of an early menopause were women treated with both radiation below the diaphragm and alkylating agent chemotherapy and by the age of 31, 42% of these women had reached the menopause compared with only 5% of controls (Byrne et al., 1992).

1.2.2 Radiotherapy

The risk of ovarian damage after radiotherapy is related to radiation dose, schedule and age at treatment. The primordial follicle is very radiosensitive and the LD_{50} has recently been revised at 2 Gy (Wallace et al., 2003). Following a radiation insult, the size of the surviving population determines the window of opportunity for fertility and time until the menopause. The depletion of primordial follicles that occurs at the

time of the insult is related to the number present, so that the younger the woman at the time of treatment the greater the number of follicles that survive, and the later the onset of the menopause. In women aged 40 years it has been shown that a permanent menopause may be induced by 6 Gy (Lushbaugh and Casarett, 1976). For younger women and children it is likely that a total dose of 20 Gy over 6 weeks would produce permanent sterility with 95% confidence.

In a study of 38 patients who received whole abdominal radiotherapy (20 –30 Gy) in childhood, all but one patient developed ovarian failure, (27 experienced pubertal failure, and a premature menopause occurred in 10 (median age 23.5 years)) (Wallace et al., 1989). The risk of ovarian failure after treatment with total body irradiation (TBI) as conditioning for a bone marrow transplant is significant but less predictable. In a large study of 718 long term survivors, treated with chemotherapy and/or TBI as conditioning treatment before a bone marrow transplant, 532 had received TBI (10 – 15.75 Gy, single exposure or fractionated) and 186 chemotherapy, with either cyclophosphamide or busulphan (Sanders et al., 1996). After TBI, 90% developed ovarian failure and after cyclophosphamide or busulphan 60% failed. There were 16 spontaneous pregnancies to 13 women who had received TBI, 6 of these women were prepubertal at the time of their radiotherapy. In a study of 16 girls treated prepubertally with TBI, 9 (56%) had spontaneous pubertal progression, although 6 of these women had increased plasma concentrations of gonadotrophins (Sarafouglou et al., 1997). The women with ovarian failure were significantly older at the time of their radiotherapy compared with female patients with spontaneous puberty (8.6 ±2.3 years vs 6.1 ± 1.8 years).

1.2.3 Chemotherapy

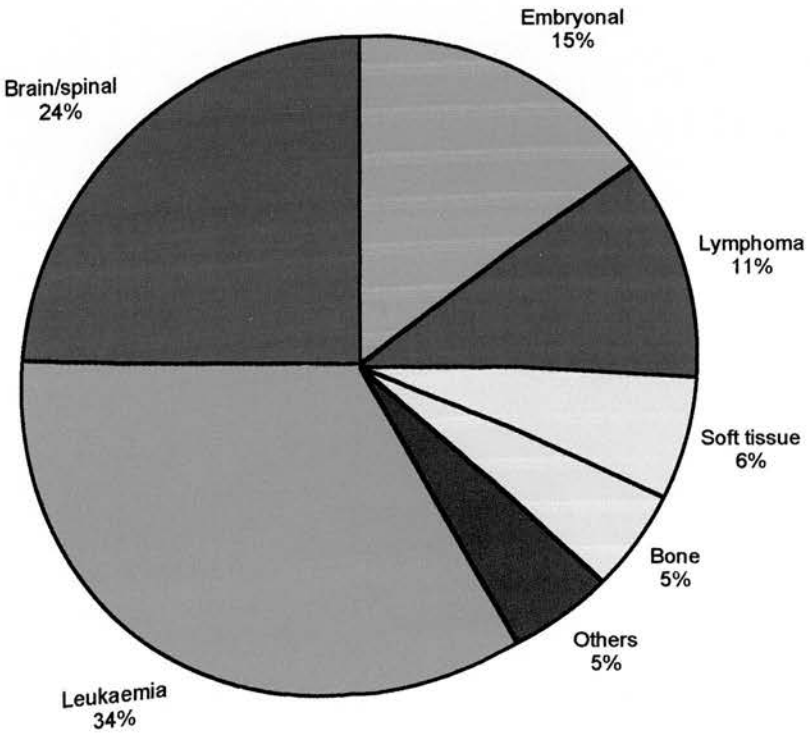
Chemotherapy causes both early and late effects which limit the dose and schedule of exposure. The impact of combination cytotoxic chemotherapy on gonadal function is dependent on the nature and total dosage of the drugs received by the child. The ovary is chemosensitive, and agents that are recognized to cause gonadal damage are listed in Table 1.1. There is a trend towards intensification of treatment, particularly with childhood leukaemia. Thus the risk of gonadal damage with these evolving regimens needs to be continually evaluated.

The majority of early reports of ovarian function in children treated with combination chemotherapy for acute lymphoblastic leukaemia suggest that premature ovarian failure is uncommon (Siris et al., 1976; Pasqualini et al., 1987; Green et al., 1989). In a UK study of 40 girls treated in childhood for ALL, all achieved adult pubertal development and 37 had regular menses. There were 14 live births in nine long-term survivors with no serious congenital abnormalities and no reported cases of malignant disease (Wallace et al., 1993). However there have been reports of premature ovarian failure in women treated for ALL with more intensive regimens. Quigley et al (1989) found significantly high basal and peak FSH levels following GnRH administration in prepubertal and pubertal girls. All the girls reached puberty at a normal time and had normal oestradiol levels. Modern treatment for childhood ALL in the UK seems unlikely to be sterilizing although these women may be at increased risk of a premature menopause.

Table 1.1 Gonadal toxic chemotherapy agents.

Alkylating agents	Cyclophosphamide
	Ifosfamide
	Nitrosoureas
	Chlorambucil
	Melphalan
	Busulphan
Vinca-alkaloids	Vinblastine
Antimetabolites	Cytarabine
Platinum agents	Cisplatin
Others	Procarbazine

Figure 1.1 Distribution of paediatric cancers.



Early reports of ovarian function following the treatment of Hodgkin's disease in childhood (Bramswig et al., 1989) suggested that ovarian function was normal in all women studied. However, a study of 32 women treated with ChlVPP (chlorambucil, vinblastine, procarbazine and prednisilone) and no radiotherapy below the diaphragm demonstrated raised gonadotrophin levels with variable oestradiol levels in 53% (Mackie et al., 1996). Seven women had achieved eleven normal pregnancies, and two of these women had raised gonadotrophins before conception. Follow up of these women is required to determine whether there is recovery of ovarian function or progression to a premature menopause.

Ovarian function in children treated with cyclophosphamide (up to 200mg/kg) as conditioning for BMT was reported as normal in 95%, higher doses in excess of 200m/kg may result in premature ovarian failure (Sanders et al., 1991). Current studies are evaluating the effect of ifosfamide on ovarian function. As all these drugs are rarely used as single chemotherapy agents, accurate assessment of the gonadal toxicity remains complex. New chemotherapy agents and multiagent chemotherapy protocols need to be continually evaluated to determine the risk of late effects.

1.2.4 Cranial irradiation

Hypothalamic - pituitary function shows progressive compromise following high dose cranial irradiation (> 30Gy). The risk relates both to the dose and whether the primary pathology involves the pituitary gland. Littley and colleagues demonstrated

that the risk of gonadotrophin deficiency was 60% 4 years out from treatment, but these subjects had pituitary adenomas as the primary pathology (Littley et al., 1989). For those patients who have received high dose cranial irradiation for tumours not involving the pituitary the risk of gonadotrophin deficiency is significantly lower, and has been reported as 2-6 % for 40 – 50 Gy, 20 – 50% for greater than 50 Gy (Gleeson and Shalet, 2004). Low dose CNS directed radiotherapy, as part of treatment for ALL, has been associated with subtle perturbations in growth hormone secretion (Crowne et al., 1992; Brennan et al., 1998) but there are few reports assessing effect on other pituitary hormone secretion in adulthood (Birkebaek et al., 1998). In a study of reproduction following treatment for childhood ALL in the Scandinavian countries, women who had received prophylactic radiation of the CNS had a significantly lower first birth rate than those without radiation, indicating that doses of 18 – 24 Gy to the brain may be a possible risk factor (Nygaard et al., 1991). For those women with confirmed hypogonadotrophic hypogondism after cranial radiotherapy, ovulation induction may be achieved with pulsed gonadotrophin therapy.

1.3 Late effects of treatment on uterine function

1.3.1 Radiotherapy

Several studies have demonstrated that uterine function is compromised by radiation exposure. Following whole abdominal irradiation (20 - 30 Gy) in childhood, all pregnancies occurring in women with preserved ovarian function resulted in mid trimester miscarriage (Wallace et al., 1989). The women with ovarian failure secondary to whole abdominal irradiation (20 –30 Gy) had a significantly reduced uterine length, which showed no improvement in size, blood flow or endometrial thickness when exposed to physiological sex steroid replacement for one month (Critchley et al., 1990). Women treated with TBI (10 – 15.75 Gy) with preserved ovarian function also have an increased risk of miscarriage, premature delivery and low birth weight (Sanders et al., 1996). Six of 16 pregnancies for 13 TBI recipients were terminated by spontaneous miscarriage. Six of the girls who were pregnant had received their treatment prepubertally. Of the six pregnancies, five resulted in spontaneous miscarriage and one an elective termination of pregnancy. There were therefore no live offspring to girls treated prepubertally. There were 8 live births, 5 of which were premature, 4 of these babies were of low birth weight and one very low birth weight.

Uterine volume post TBI has been assessed during standard hormone replacement and has been shown to be significantly reduced compared to controls (Holm et al., 1999). A successful pregnancy outcome for women whose uterus has been exposed

to radiotherapy prepubertally may be compromised. Women treated post pubertally were noted to have a larger uterine volume and viable pregnancies have been reported for this group. The effects of radiation are thought to be both on the uterine musculature, with fibrosis and loss of distensibility, and on uterine artery blood flow. For women with premature ovarian failure the potential for pregnancy has been made a realistic possibility with ovum donation and IVF technologies. Pregnancy has been achieved for women who have ovarian failure secondary to radiation exposure. The only live offspring reported are to women whose uterus has been exposed to radiotherapy post pubertally. Oocyte donation achieved conceptions in 2 of 3 women who were treated with TBI for a haematological malignancy; one woman with a uterus of almost normal size, who was treated postpubertally, delivered a healthy girl at term and one woman with a small uterine volume, who was treated pre/peri pubertally, conceived but had a spontaneous mid trimester miscarriage (Larsen et al, 2000).

1.3.2 Chemotherapy

There is no evidence that chemotherapy has a significant long term effect on uterine function. There are no reports in the literature of an increased risk of miscarriage and there are many reports of normal pregnancy outcome following multi agent chemotherapy protocols (Quigley et al., 1989; Nicholson and Byrne, 1993).

1.4 Conclusion

The identification of late effects of the treatment of childhood cancer enables future physicians to pursue the ethos of achieving a cure while minimizing the risk of long term effects from the treatment. With the intensification of treatment strategies children will remain at risk of infertility, secondary to the therapies employed. Evaluation of late effects and novel strategies for preserving reproductive potential enables informed best practice for preserving the reproductive potential for children who require treatment for cancer.

CHAPTER 2: Ovarian and uterine function – normal physiology and investigation of function

2.1 Introduction

In the post pubertal female, tightly coordinated functions between the hypothalamus and pituitary gland, the ovary and the endometrium give rise to cyclical predictable menses that indicate regular ovulation. An understanding of the normal physiology is essential in investigating females who have been exposed to therapies that may affect this complex process.

2.2 Normal Physiology

2.2.1 Background

The evolution of this process starts with germ cell migration. The premeiotic germ cells arrive at the genital ridge by the 5th week. The number of germ cells increases and peaks at 20 weeks gestation with 6 to 7 x 10^6 follicles. There is then an inexorable decline, secondary to follicle atresia, with 1 to 2 x 10^6 follicles present at birth, 300,000 by the onset of puberty, and less than 1000 when the menopause occurs. There is therefore, a relationship between age and number of follicles in human ovaries (Block., 1952), with an accelerated decline in follicle number from age 35 (Faddy et al., 1992). Of these follicles only 400 – 500 will ovulate during the reproductive lifespan.

The ovary is made of the outer cortex and inner stroma. The outer cortex consists of the germinal epithelium and the follicles. Each ovarian follicle comprises an egg, the oocyte, surrounded by granulosa cells, which in turn are surrounded by theca cells. The follicle is the functional unit of the ovary. The majority of the follicles are in a non growing state, termed the primordial follicle. The recruitment of primordial follicles to primary follicles is controlled by local ovarian factors, and continues independently of hypothalamic pituitary control. The follicle matures from primordial to a primary and then a secondary follicle – the preantral stage. Under gonadotrophin control, the follicle further matures to an antral follicle. This is

therefore only possible once the hypothalamic pituitary gonadal axis has matured through puberty. Post puberty, the ovary has two main objectives, to produce fertilisable ovum and prepare the endometrium for implantation.

Plasma levels of LH and FSH rise in the fetus after establishment of the hypothalamic pituitary portal system, until mid gestation, and then fall towards term as inhibitory influences, the sex steroids, rise towards term. During the first 2 years of life, plasma levels of LH and FSH rise intermittently, and from mid childhood the plasma levels remain low until puberty. Puberty involves changes in the central nervous system and an increase in the frequency and amplitude of LHRH secretion, which initiates and regulates the secretion of pituitary gonadotrophins and gonadal sex steroids that promote development of secondary sexual characteristics. There is an increase in the amplitude of LH and FSH secretion at night from about 5 years of age. The amplitude and frequency of these peaks increase and daytime secretion increases with the progression of pubertal development.

The major oestrogen in females is oestradiol, which is prinicipally secreted by the ovary. In the fetus and at term, oestradiol levels are high because of conversion of the fetal and adrenal C19 steroids to oestradiol by the placenta. The plasma levels drop precipitously in the first few days of life. Levels remain very low until the onset of puberty. Studies with ultra sensitive assays have shown gradually increasing levels of oestradiol prior to the clinical onset of puberty, consistent with the gradual increase in FSH. In puberty oestradiol in secreted by the granulosa cells, under

control of FSH. LH stimulates androstenedione production from the theca cells which is peripherally converted to oestradiol.

Progesterone is the principal secretory product of the corpus luteum, and is responsible for the progestational effects of cell differentiation and the induction of secretory activity in the endometrium. Progesterone is therefore required for implantation of the fertilised ovum and maintenance of pregnancy.

Inhibin is a peptide produced by the granulosa cells, and has a major regulatory role in FSH production by the pituitary. Inhibin is a heterodimer, composed of common α subunit and different β subunits, denoted β_A and β_B, termed A and B respectively. Both isoforms have similar properties, but synthesis is regulated differently throughout the cycle. Inhibin B is secreted during the early follicular phase, decreasing in the mid follicular phase, and is undetectable after the LH surge. Inhibin A is low during the first half of the follicular phase, and increases during the mid follicular phase with a peak during the luteal phase. Levels are low during childhood, and the positive relationship with FSH indicates that sporadic follicular development through infancy and childhood is under the influence of FSH. Both increase during puberty and the relationship to FSH changes from mid puberty, to that seen in post puberty (Crofton et al., 2002)

Onset of menses occurs with complex interaction of sex steroids, ovarian peptides and gonadotrophins. Increasing FSH allows for follicle growth and development, and increasing oestradiol secretion. The mid cycle surge of LH stimulates rupture of

follicle and release of egg, and formation of the corpus luteum. Progesterone and oestradiol are secreted by the corpus luteum. Oestradiol induces growth of the endometrium and progesterone enhances differentiation. Sloughing of the endometrium follows withdrawal of oestradiol and progesterone as the corpus luteum involutes in the absence of pregnancy.

2.3 Investigation of hypothalamic and ovarian function

2.3.1 Biochemical assessment

In infancy the hypothalamic – ovarian axis is active and gonadotrophin levels are detectable. However, these levels fall, and remain low until the onset of puberty. The activity of the hypothalamus, and the production of LH and FSH can be investigated with stimulation tests, that are principally employed to detect precocious or premature puberty in young girls (Zevenhuijzen et al., 2004). Biochemical detection of gonadal damage is not reliable in prepuberty, although stimulation tests may reveal a brisk FSH response, in late prepuberty, in those with ovarian failure. Elevated baseline gonadotrophin levels, as the early manifestation of ovarian failure, may be detectable in infancy, as may be seen in young girls with Turners syndrome, or when a child is of an age and physical maturity appropriate for the onset of puberty. Failure of development of secondary sexual characteristics at an age appropriate time, with rising gonadotrophins are suggestive of ovarian failure. Elevated gonadotrophins, undetectable oestradiol and failure of pubertal progression indicate ovarian failure and the need for oestrogen replacement for pubertal induction, progressing to cyclical hormone replacement post pubertally.

Detection of subtle hypothalamic/pituitary ovarian dysfunction and prediction of a premature menopause are difficult. Early follicular phase assay of FSH, oestradiol and inhibin B are potential tools to assess ovarian reserve. While there are few direct

data on changes in these markers in the context of chemotherapy or radiotherapy, there is considerable evidence for their value in detecting the changes associated with normal ageing, and as predictors of the ovarian response to superovulation which is believed to be an index of the total follicular pool (Cruz et al., 1996). A rise in early follicular phase FSH with maintained oestrogen production is well recognised to be a feature of the perimenopause (Sherman et al., 1976; Reyes et al., 1977), and is detectable from approximately 20 years before the menopause (Ahmed Ebbiary et al., 1994). The pattern of secretion of inhibin B across the menstrual cycle is consistent with secretion by the developing cohort of follicles, while inhibin A is predominantly secreted by the dominant follicle (Groome et al., 1994; Groome et al., 1996; Welt et al., 1997). Inhibin B concentrations are reduced in the early follicular phase in older women (Klein et al., 1999; Welt et al., 1999) and are correlated with the ovarian response to exogenous gonadotrophin stimulation (Seifer et al., 1997; Hall et al., 1999; Eldar-Geva et al., 2000). Inhibin B appears to be the earliest endocrine marker of the perimenopause (Burger et al., 1998).

2.3.2 Radiological assessment

Imaging of the ovary and uterus through childhood and puberty has shown changes consistent with biochemical assessments, and parallels the clinical findings of puberty as described by Marshall and Tanner (Marshall et al., 1976). Pelvic ultrasound has proved to be an accurate, painless and non-invasive investigation in the assessment of internal genitalia. Uterine and ovarian growth are correlated both

with age, in the prebubertal child, and pubertal stage (Herter et al., 2002). A cross-sectional study of 166 girls aged 6 – 18 years, investigated ovarian and uterine shape and morphology in relation to pubertal stage (Holm et al., 1995). The appearance of breast development was preceded by growth and development of the internal female genitalia. The median (range) ovarian volume in the prepubertal girls was 1.2 mls (0.5 – 5.1 mls). Ovarian growth was most pronounced between breast stages 2 and 4. Median ovarian volumes were 2.2mls, 4.1 mls, 6.2 mls, and 7.3 mls for puberty stages 2,3,4 and 5 respectively. Follicle activity was seen in 87% of prepubertal girls. From the age of 14-15 years, the size of follicles began to increase and vary considerably, reflecting that girls were in different stages of the menstrual cycle. Ovarian volume has been shown to decline with age in the post pubertal women (Wallace et al., 2004). Other workers have demonstrated that antral follicle count showed the best correlation with women's age, and declined linearly at a rate of 3.8% per year (Ng et al., 2003). Ovarian volume has been shown to predict the number of recruitable follicles during super ovulation (Syrop et al., 1999).

2.4 Investigation of uterine function.

2.4.1 Radiological assessment

Ultrasound scanning is a reliable non-invasive technique for assessing uterine size and shape, blood supply and endometrial thickness. The uterine shape changes through puberty and is a useful indicator of pubertal stage. Normal uterine length and volume for pre, peri and post puberty are well documented (Holm et al., 1995). In this study, uterine volume increased before clinical evidence of onset of puberty. The volume continued to increase through puberty with the greatest increase in size occurring between Tanner stages 3 and 4. The gains achieved after menarche were also significant, with uterine volume increasing up to age 20 years. No correlation was found between uterine volume and the height of the girls. The uterine volumes were 1.6 (0.7 – 7.9) mls, 2.8 (1.3 – 8.1) mls, 8.0 (2.0 – 18) mls, 37 (11 - 56) mls, 43 (12 – 82) mls and 61 (37 – 130) mls for Tanner breast stages 1, 2, 3, 4, 5 and adult respectively. A study of young girls with Turner syndrome documented normal uterine growth in those with preserved ovarian function, but in those who required pubertal induction with exogenous steroids, 50% did not achieve normal adult uterine dimensions (Paterson et al, 2002).

Uterine artery blood flow may be assessed by Doppler scanning and quantified as the pulsatility index indicating degree of resistance to flow, distal to the point of sampling (Taylor et al, 1985; Steer et al, 1990). Diastolic flow has been

demonstrated in 35% of prebubertal females and 100% of normal adult women with regular menses (Laursen et al, 1996).

2.4.2 Histological assessment

Endometrial biopsy may be performed as an out patient using a Pipelle endometrial sampler that obtains a piece of the endometrium by aspiration (Sauer et al., 1997; Critchley at al., 2004). It can be performed without cervical dilatation and is therefore usually associated with minimal discomfort. Endometrial function may then be assessed by histology (Noyes et al., 1950) and with immunno-histological techniques (Snidjers et al., 1992; Critchley et al., 1998).

CHAPTER 3: Aims of thesis

3.1 Aims

The purpose of this thesis is to investigate the long term effect of cytotoxic treatment (radiotherapy and/or chemotherapy) on the reproductive health of women treated for childhood cancer. Long term survivors were studied to help elucidate these issues.

The hypotheses were that

1. there are subtle effects on hypothalamic and ovarian function following non sterilising therapy for childhood cancer.
2. ovarian reserve can be predicted using biochemical and radiological investigation
3. the uterus is significantly affected by radiotherapy but parameters can be improved by optimising hormone replacement therapy
4. there is no consensus on hormone replacement therapy for young women with ovarian failure

The majority of women are treated with non-sterilising therapy that may have a significant effect on hypothalamic or ovarian function that is only clinically apparent when they present with infertility, irregular menses or premature ovarian failure. Investigation of the risk of hypothalamic or ovarian effects and tests to aid in determining reserve would therefore be extremely valuable in elucidating the risk for these women. The effect of low dose cranial irradiation (18-24 Gray) on gonadal function was evaluated in long-term survivors of childhood leukaemia. Ovarian

reserve was assessed in women who had been treated with chemotherapy in childhood. We assessed women with regular menstrual cycles and women with a history of regular cycles who were using the COCP for contraception. A significant number of women use the COCP and there is no literature regarding ovarian assessment while on the COCP. They were investigated before and 24 hours after an injection of FSH to assess baseline ovarian function and in response to stimulation.

For those women with ovarian failure secondary to radiotherapy to the pelvis, the effect on the uterus is not well characterised. Optimising oestrogen and progesterone replacement may improve uterine function. Ovarian and uterine function were evaluated in women who had received total body irradiation in childhood (14.4 Gray). In women with ovarian failure, uterine function was evaluated before and after 3 months of pSSR.

For those young women who have ovarian failure there is no good evidence as to the optimal method of pubertal induction and subsequent cyclical hormone replacement therapy to maximise skeletal, cardiovascular, reproductive and psychological well being. UK practice was evaluated to determine whether there was a consensus.

A case is reported of a girl who had clinical and biochemical evidence of ovarian failure after treatment with pelvic radiotherapy and chemotherapy. The case elucidates the difficulties in biochemical assessment of ovarian failure, optimising hormone replacement therapy and accurate advice regarding fertility potential.

CHAPTER 4: Hypothalamic – Pituitary – Ovarian dysfunction after prepubertal chemotherapy and cranial irradiation for acute leukaemia

4.1 Introduction

The majority of children presenting now with ALL will be cured (Chessels et al., 1995). In the 1960s the cure rate was less than 5%. Increased survival over the following twenty years was achieved with chemotherapy and radiotherapy and by the 1980s the survival rate had risen to 70%. This allowed a shift in aim over the last 20 years to reducing treatment related effects while further improving the survival rate.

While high dose cranial radiation is recognised to significantly compromise hypothalamic – pituitary function, the effects of the relatively low dose irradiation for childhood leukaemia are uncertain. Reports of ovarian function after treatment of standard risk childhood leukaemia have been reassuring (Wallace et al., 1993), however the relatively recent advances in treatment, and thus survival mean that few patients are beyond their 30^{th} birthday. We have therefore carried out a detailed investigation of hypothalamo-pituitary-ovarian function in long-term survivors of childhood ALL.

4.2 Materials and methods

Women were eligible for recruitment to this study if they were in first clinical remission following treatment for ALL (chemotherapy with cranial irradiation) in childhood or early adolescence, five or more years out from end of treatment and were post-pubertal at the time of recruitment. Thirty such women were identified from the long-term follow up clinic at The Royal Hospital for Sick Children, Edinburgh. Eighteen were excluded from assessment: one woman was pregnant, 8 were using hormonal contraception, 7 declined to take part and 2 had ongoing medical problems. Twelve women were therefore recruited for participation in the study. Sixteen healthy women with regular menstrual cycles were recruited as normal controls. They were recruited by two methods: a) a poster campaign requested women who had regular menses, were not on the OCP and aged 18 – 30 years and were interested in taking part in a research study to contact the study coordinator and b) by a special study module student (a research module during 4th year of undergraduate training) who approached friends who were also undergraduates, regarding participation in a research study.

The combination chemotherapy schedules were the Medical Research Council studies UKALL I, II, V, VIII and X. All these protocols included the use of vincristine, 6 mercaptopurine, prednisolone and methotrexate. Other drugs used in some schedules included asparaginase, cytosine arabinoside, doxorubicin and cyclophosphamide. The protocols evolved with increasing intensity of chemotherapy

with subsequent protocols. All patients received cranial irradiation in a total dose of 18 – 24 Gy, depending on the protocol, and 2 patients received in addition spinal irradiation (dose 10-14 Gy), which has been documented to have a direct effect on ovarian function in some patients.

4.2.1 Subjects details

The 12 women recruited following treatment for ALL had median age at diagnosis of 4.7 years (1.9 – 13.1), and at assessment 20.8 years (15.8 – 32.8) (see table 4.1). Mean BMI was 25.8±1.2kg/m^2. The controls were aged 17.3 – 29.0 years (median 24.1), with regular menstrual cycles (25-32 days) and had not used hormonal contraception in the preceding 3 months. Mean BMI was 23.3±1.2kg/m^2. A full menstrual and pregnancy history was taken from subjects and controls, and all were confirmed to be euthyroid and have normal prolactin concentrations.

The local ethics committee gave approval for the study and informed consent was obtained from all women.

4.2.2 Study design

The women collected a daily early morning urine sample from day 1 of the cycle for a minimum of 2 cycles in ALL patients (total of 41 cycles) and 1 cycle in controls.

Table 4.1 Patient details

Patient	Diagnosis	Age at diagnosis	Treatment	CI Gray	Spinal Irradiation	Age at assessment
1	ALL	3.2	UKALL VII	1800	N	16.3
2	ALL	3.9	UKALL I	2500	1000	30.1
3	ALL	1.9	CHOP	1800	N	22
4	ALL	5.5	UKALL X	1800	N	17.3
5	ALL	2.1	UKALL II	2400	N	19.5
6	ALL	11.2	UKALL II	2400	1000	32.6
7	ALL	3.2	UKALL VII	1800	N	16.8
8	ALL	6	UKALL V	1800	N	23.2
9	ALL	10	UKALL X	1800	N	17.3
10	ALL	7.2	UKALL V	1800	N	23.7
11	ALL	2.2	UKALL V	1800	N	16
12	ALL	13.1	UKALL VII	1800	N	24.3

Preliminary data suggested that cycle characteristics were more variable in ALL patients than controls, thus repeated cycles were collected and analysed where possible. At least 3 cycles were collected by 9 of the ALL patients. While control women initially collected 2 consecutive cycles, in view of the consistency of cycle characteristics in these women, only 1 cycle per woman was included in the detailed analysis to give the normal comparison group.

All women also attended during the early follicular phase (day 3-5) of a cycle for blood sampling and ultrasound scan of the ovaries. Blood samples were analysed for plasma E_2, P4, LH, FSH, inhibin A and B, IGF1 and IGFBP3 and prolactin. All ultrasound examinations were performed by one of two radiologists trained in gynaecological scanning using a Hitachi EUB 555. Scans were performed transvaginally (6.5 MHz transducer) in sexually active women and transabdominally (3.5 MHz transducer) in those who were not. Ovarian volume was measured in three orthogonal diameters and using the formula for a prolate ellipsoid ($d_1 x d_2 x d_3$) x 0.523 (Holm et al., 1996).

4.2.3 Hormone analyses

Urine samples were analysed for luteinising hormone (LH), oestrone conjugates (E1C) and pregnanediol-3-glucuronide (P3G). Urinary E1C and P3G were measured using an "in house" ELISA using HRP-conjugate as label and solid-phase second antibody separation (for E1C, CV < 4%; for P3G, CV <13%). Urinary LH was

measured by a two-site IRMA (Serono MAIA clone, CV < 12%). Urinary hormone concentrations were corrected for creatinine concentration. Serum E2 was measured by competitive immunoassay using the Boehringer Mannheim Elecsys (Mannheim, Germany). P4, FSH and LH were measured by microparticle enzyme immunoassay on the Abbott Axsym (Chicago, Illinois). Inhibin A and B were measured by two-site ELISAs as previously reported (Groome et al., 1994). For inhibin B assay sensitivity was 7.8 pg/ml, intra- and inter-plate CVs were 10.6% and 11.4% respectively and for inhibin A intra- and inter-plate CVs were 5.0% and 12.7% respectively. IGF1 and IGFBP3 were measured as previously described (Blum., 1996).

4.2.4 Statistical analysis

The day of onset of menses was defined as cycle day 1, and the last day of the cycle was that preceding the next menstruation, giving cycle length. The last day of the follicular phase was the day of the onset of the LH surge, defined as a minimum of a 2.5-fold rise in LH excretion over the mean LH concentration in the preceding 4 days. Follicular and luteal steroid excretion was calculated as the integrated area under the curve using the trapezoid method over the relevant part of the cycle (total follicular phase, days 2-5 and days 6-12 to give measures of early and late follicular phases, and total luteal phase). Peak luteal P3G was calculated as the mean of the 3 consecutive days of maximal excretion, and the LH surge was quantified as the sum of values of the 3 days from the onset of the LH surge. Follicular LH excretion was calculated as the mean of the first 7 days of the cycle.

Hormonal data are presented as mean ± sem. Data were log transformed to correct non-equality of variance and compared by ANOVA and Student's t test. Initial comparisons investigated differences between controls and all ALL women's cycles. If this indicated a significant difference, data were further analysed by ANOVA after subdivision of ALL patient cycles into those with normal or short luteal phases, cycles with a luteal phase length of ≤ 11 days being defined as short. Data showing significant non-equality of variance (cycle characteristics and ovarian volume) were analysed using the Mann-Whitney test.

4.3 Results

4.3.1 Cycle characteristics

All 12 women treated for childhood ALL had achieved adult sexual development and the onset of menses. They reported regular menstrual cycles (26 - 30 days), and this was confirmed during the study when ovulatory cycles were demonstrated in all subjects. None had been pregnant. A total of 41 cycles from ALL patients were analysed (table 4.2). 2 cycles from ALL patients were anovulatory (no LH surge and no rise in P3G), both in women who also had normal ovulatory cycles. These 2 cycles were excluded from further analysis. No anovulatory cycles were seen in the control women.

Overall cycle length was not different in ALL patients (table 4.2) compared to controls (table 4.3) (28.1±0.5 vs. 28.7±0.7 days, figure 4.1). Length of the follicular phase was also similar (15.9±0.4 vs. 15.2±0.6 days) but the length of the luteal phase was significantly shorter in ALL patients (12.2±0.3 vs. 13.6±0.4 days, $p=0.01$). Closer analysis of luteal phase length indicated a high prevalence of short (≤11 days) luteal phases in ALL patients, whereas only 1 such cycle was seen in controls. Overall, 15 out of 39 apparently ovulatory cycles in ALL patients showed short luteal phases, in 5 of the 12 patients. Short luteal phases were not always consistently seen in those ALL patients who demonstrated them: all 4 cycles from

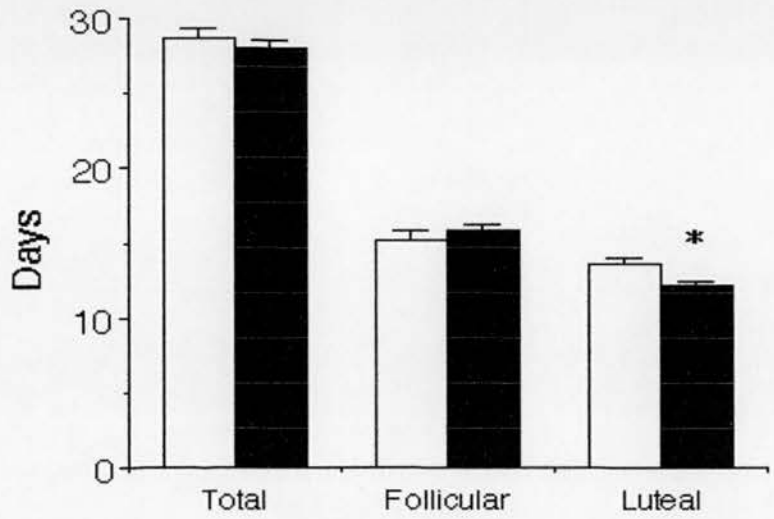
Table 4.2 Details of number of completed cycles each patient collected urine samples and each cycle length and length of luteal phase in days

Pt no	No of cycles	Cycle length	Luteal length
1	2	29,27 (1 anov)	13,12
2	4	28,35,31,25	15,14,14,14
3	3	27,28,26	12,11,12
4	1	30 (1 anov)	14
5	5	27,25,26,25,27	10,10,10,12,11
6	4	22,26,25,25	8,9,10,10
7	2	36,35	11,13
8	5	28,28,29,30,26	10,12,16,13,10
9	3	27,34,30	13,15,15
10	5	26,24,24,30,26	13,11,12,13,11
11	3	34,31,29	14,14,13
12	2	128,26	12,11

Table 4.3 Details of the cycles of the control women during which urine samples were collected

Subject	Cycle Length	Luteal length
1	31	15
2	27	13
3	29	13
4	31	14
5	28	15
6	29	14
7	30	14
8	27	13
9	30	14
10	26	11
11	27	13
12	28	14
13	28	12
14	30	13
15	29	12
16	27	14

Figure 4.1 Cycle length in controls (open bars) and ALL subjects (filled bars). Total cycle length, and duration of the follicular and luteal phases. * p=0.01. n=16, control; n=12, ALL subjects, mean ± sem.



one subject showed short luteal phases, but cycles with normal luteal length were also seen in the other 4 patients. These cycles are hereafter referred to as 'short' and 'normal' ALL cycles in distinction to control cycles (i.e. in normal, control women).

ALL patients with short luteal phases did not differ in age at initial treatment or age at present investigation from those who only showed normal luteal phase length (figure 4.2). There was however a significantly greater interval between initial treatment and time of present investigation in those showing short luteal phases ($p=0.04$), consistent with a progressive effect of treatment. BMI did not differ between the two groups (26.1 ± 0.6 kg/m^2, normal cycle group; 25.4 ± 1.9 kg/m^2, short luteal cycle group).

4.3.2 Urinary hormone excretion

Detailed analysis of daily urinary hormone excretion revealed several differences between ALL patients and controls. During the follicular phase, LH excretion was lower in ALL patients than controls ($p=0.002$, figure 4.3a), with lowest levels in short luteal phase cycles which were significantly different both from controls ($p=0.005$) and ALL cycles with normal luteal phase length ($p=0.03$). Normal length ALL cycles had lower LH levels than control cycles ($p=0.05$). A more marked difference in LH excretion was seen during the LH surge (figure 4.3b). Mean 3 day LH excretion was significantly lower in ALL cycles than controls ($p<0.0001$), with both short and normal ALL cycles showing this difference ($p=0.0005$ and $p=0.004$

Figure 4.2 Age at initial treatment, present investigation, and interval since treatment in ALL patients who showed cycles with short luteal phases (n=5, open bars) and those who only showed cycles with normal luteal phases (n=7, hatched bars). Mean ± sem.

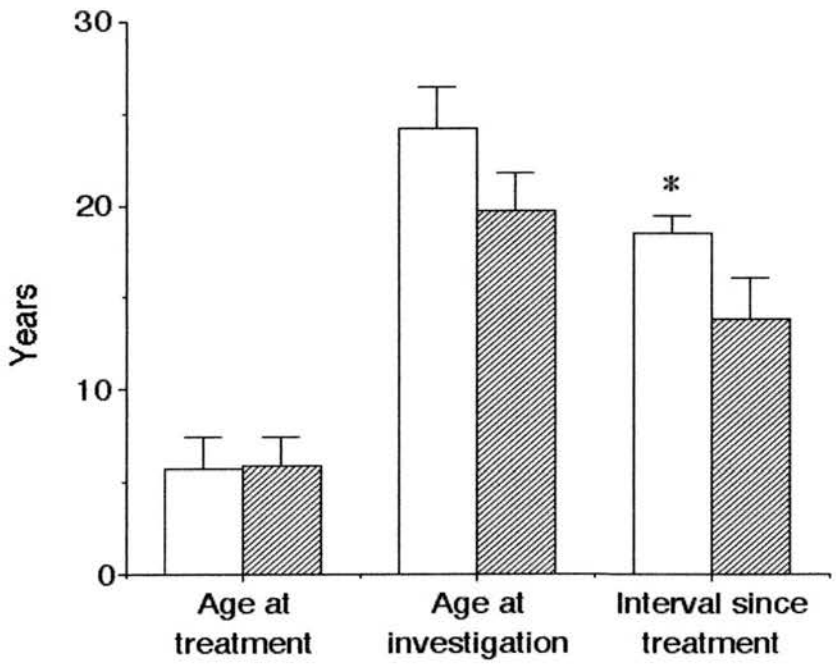
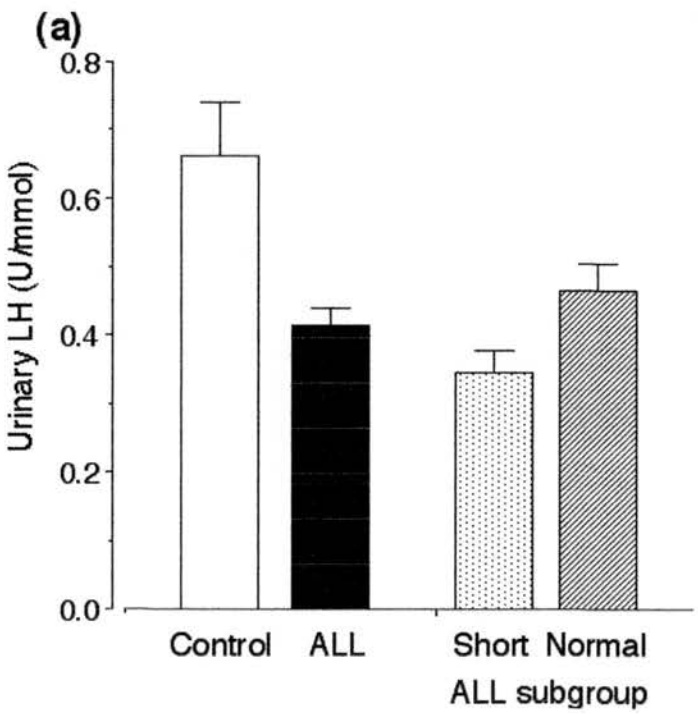
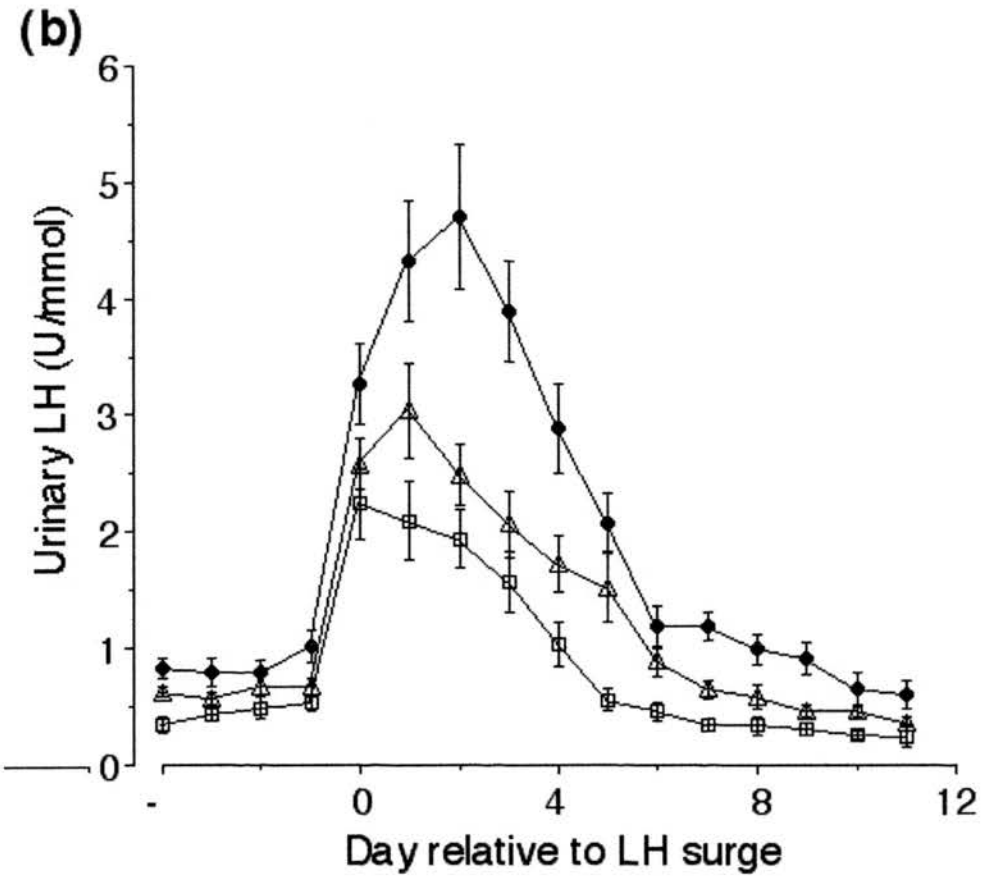


Figure 4.3 Urinary LH excretion in control and ALL subject cycles.

(a) Mean day 1-7 LH excretion in controls (open bars, n=16) and ALL subjects (filled bars, n=39). The stippled and hatched bars represent LH excretion in the ALL subgroups of cycles with short (n=15) and normal (n=24) luteal phase length respectively. * $p<0.05$; ** $p<0.002$ vs control cycles; † $p<0.05$ short vs normal ALL cycles.



(b) Urinary LH excretion in control and ALL subject cycles. Daily LH excretion centred on the day of onset of the LH surge (cycle day 0) in control cycles (filled circles, n=16) and cycles or ALL subjects with normal (open triangles, n=24) and short (n=15) luteal lengths. Mean ± sem.



respectively vs control). There was no significant difference between normal and short ALL cycles in this respect. LH excretion remained lower in ALL patients than controls throughout the luteal phase (figure 4.3b).

Follicular phase E1C excretion during both early (days 2-5) and late (days 6-12) follicular phase was lower in ALL cycles ($p=0.01$ in both cases, figure 4.4a and 4.4b). Reduced excretion was only lower than control in normal ALL cycles ($p=0.007$ and $p=0.001$, early and late follicular phases) whereas there were no differences between control and short ALL cycles. E1C excretion was lower in normal than short ALL cycles at both stages of the follicular stage ($p=0.02$ in both cases).

Luteal phase excretion of E1C was also lower in ALL cycles that controls ($p=0.01$) (figure 4.4c). Reduced excretion of E1C was detected in both normal and short ALL cycles compared to controls ($p=0.04$ and $p=0.03$ respectively), but there was no significant difference between normal and short ALL cycles. Luteal phase P3G was slightly but not significantly lower in ALL cycles than controls, both when analysed as a 3 day peak or total luteal phase excretion (figure 4.4d).

4.3.3 Plasma hormones

Blood samples taken during the early follicular phase of the cycle were analysed for LH, FSH, E2, inhibin A and inhibin B concentrations (tables 4.4 and 4.5). LH and

Figure 4.4 Urinary steroid excretion in control and ALL subject cycles. (a) Early and (b) late follicular phase E1G; (c) luteal phase E1G; (d) luteal phase P3G. Controls (open bars, n=16), ALL subjects (filled bars, n=39). The stippled and hatched bars represent LH excretion in the subgroups of ALL patient cycles with short (n=15) and normal (n=24) luteal phase length respectively. ** $p<0.01$ vs control; † $p<0.05$ vs short luteal length.

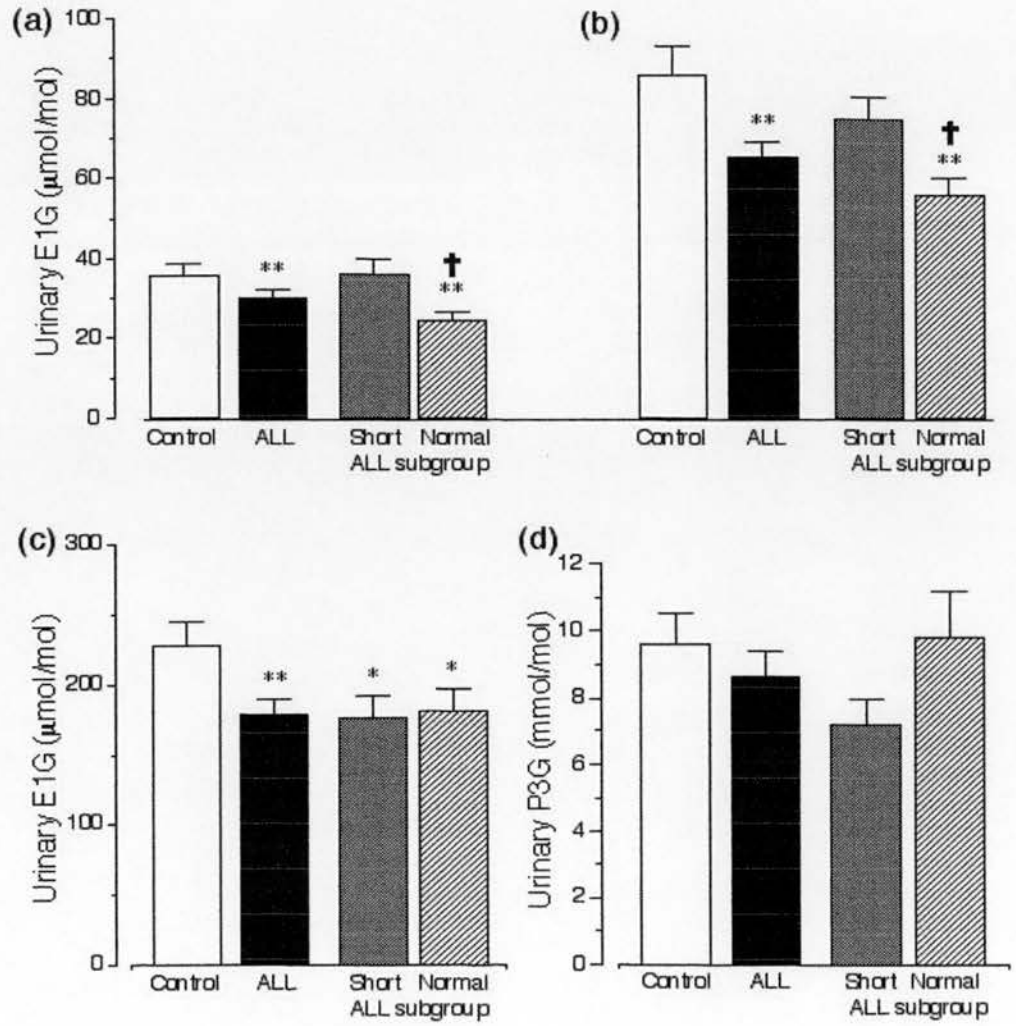


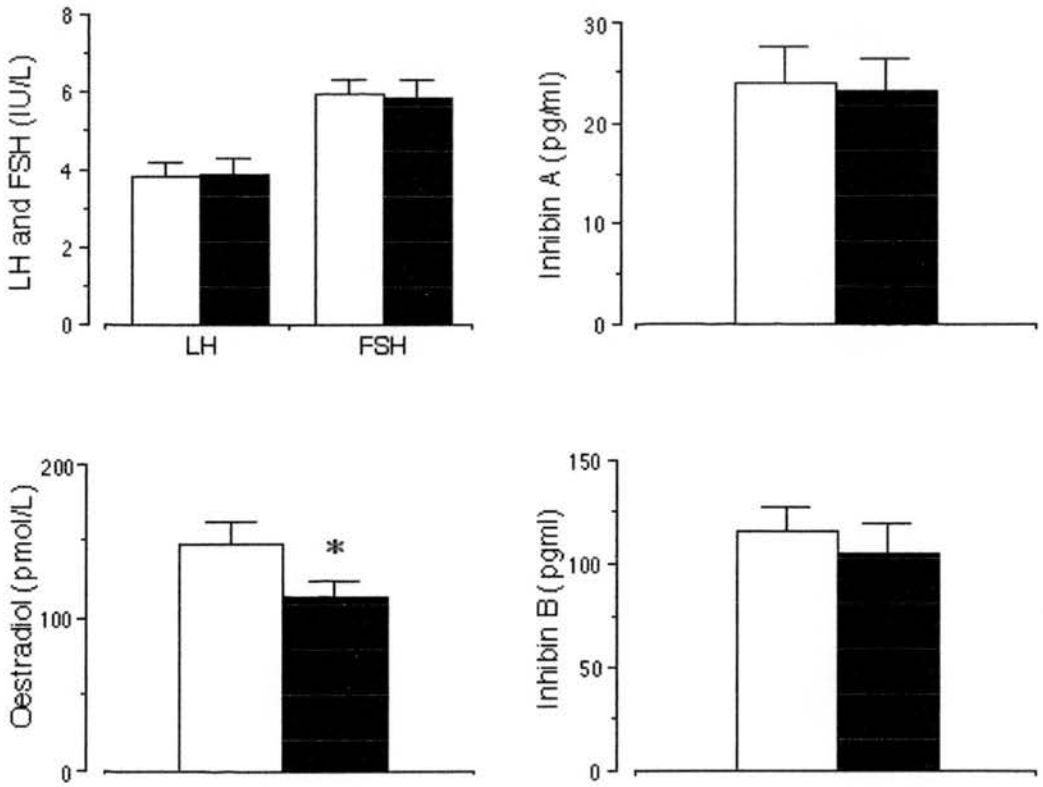
Table 4.4 Biochemical results of patients on day 3-5 of cycle

	FSH U/L	LH U/L	Oestradiol pmol/l	Inhibin B pg/ml
1	6.8	5.2	118	77.5
2	6	2.6	152	128.6
3	6.2	3.2	82	51.8
4	5.8	3.5	169	114.5
5	5.6	4.1	169	138.3
6	4.9	2.9	90	65..8
7	5	4.7	125	130.6
8	4.2	2	91	28.7
9	4	3.4	116	84
10	10.3	4.4	71	82.2
11	5.4	2.6	99	90.9
12	5.9	7	82	110.2

Table 4.5. Biochemical results of controls on day 3-5 of cycle.

	FSH U/L	LH U/L	Oestradiol pmol/l	Inhibin B pg/ml
1	3.7	2.3	110	32
2	4.7	6.5	86	156
3	5.2	6.2	305	165
4	4.9	4.6	188	214
5	8.3	4.2	275	277
6	6.1	3.9	267	106
7	6	3.7	113	104
8	5.9	4.1	128	175
9	7.8	2.7	147	72
10	7	2.9	136	49
11	7.2	3.1	132	130
12	5.9	3.8	98	108
13	7.8	3.9	156	91
14	3.4	4.6	97	87
15	5.7	5.4	156	59
16	5.8	3.9	117	99

Figure 4.5 Early follicular phase serum hormones in control and ALL subjects. Controls, open bars, n=16; ALL subjects, filled bars, n=12. * $p<0.05$ vs control. Mean ± sem.



FSH concentrations were similar in ALL patients to controls (figure 4.5a), but E2 was lower in ALL patients than controls ($p=0.032$, figure 4.5b). Inhibin A and B were similar in ALL patients and controls (figure 4.5c and 4.5d). IGF1 and IGFBPs were compared to published reference ranges (Blum., 1996) and were not significantly different from the reference range.

4.3.4 Ovarian volumes

Mean ovarian volume in the ALL group was 4.8±0.54 ml, which was not significantly different from that in controls (5.4±0.57 ml). Ovarian morphology was normal in all subjects.

4.4 Discussion

The treatment of childhood cancers frequently involves administration of drugs and radiation with potential adverse effects on reproductive function. There are few published data regarding long term follow up of fertility after standard treatment for ALL (Wallace et al., 1993; Nygaard et al., 1991). While fertility and successful pregnancies have been reported in patients after the treatment of childhood ALL with standard MRC regimens (and indeed 3 of the 30 patients identified but not studied here had had successful pregnancies), this does not rule out a significant and progressive effect on reproductive function. In a study of reproduction following treatment for childhood ALL in the Scandinavian countries, women who had received prophylactic radiation of the CNS had a significantly lower first birth rate than those without radiation, indicating that doses of 18 – 24 Gy to the brain may be a possible risk factor (Nygaard et al., 1991). Detailed analysis of endocrine function at long term follow up has not been previously reported.

We hypothesised that chemotherapy might have reduced the number of primordial/primary follicles in the ovary of patients with ALL. This was assessed by early follicular phase hormone assay and by ultrasound measurement of ovarian volume. ALL patients did not show higher FSH concentrations or lower inhibin A and B concentrations than controls, or have smaller ovaries. These results therefore appear to be reassuring as to the number of primordial follicles remaining in the ovaries of these young girls after chemotherapy for ALL. The lack of sensitivity of

the available markers and very limited longitudinal data (Welt et al., 1999) suggests, however, that such reassurance should be limited to stating that there was no evidence of any subjects being in the peri-menopause. Further longitudinal data are required to assess the predictive value of these apparently normal results in this context.

By contrast, daily analysis of urinary hormone excretion provided clear evidence of abnormal reproductive function in some ALL patients. The great majority of monitored cycles in ALL patients were ovulatory, based on the presence of an LH surge and a rise in P3G excretion. This was consistent with the regular menses reported by these patients. However LH excretion was reduced in ALL patients throughout the cycle, most markedly during the LH surge. There was also a high prevalence of cycles with short luteal phases in the ALL patients. While we have no direct evidence that this is related to reduced LH secretion in these patients, the LH surge was most deficient in cycles with short luteal phases. It is well recognised that the function of the corpus luteum is dependent on LH secretion (Hutchison et al., 1984) and in particular the magnitude and duration of the LH surge (Zelinski-Wooten et al., 1991). The apparent variability in the prevalence of short luteal phases between and within subjects (although based on small numbers) is consistent with the observed partial reduction in the magnitude of the LH surge rather than a more complete inability to mount the surge. Furthermore, the interval between treatment and investigation was longer in those patients showing short luteal defects, consistent with a progressive effect. Pituitary function shows progressive compromise following cranial irradiation in higher dose than received by the patients

described here (Littley et al., 1989). Low dose central nervous system directed radiotherapy, as part of treatment for ALL, has been previously associated with perturbations in growth hormone secretion (Crowne et al., 1992; Brennan et al., 1998) but there are no previous reports of an effect on other pituitary hormone secretion in adulthood (Birkebaek et al., 1998).

Growth hormone (GH) is the primary stimulator of the synthesis of insulin-like growth factor 1. GH insufficiency has been associated with decreased fertility (Pellicer et al., 1994). IGF1 may amplify the effects of gonadotrophins on ovarian tissue (Barreca et al., 1993) and has been postulated to have an effect on uterine receptivity (Potashnik et al., 1995). In this study, levels of IGF1 were within the normal range and did not show a correlation with time since treatment. These data do not suggest a significant GH deficiency in these patients. However, subtle defects in GH secretion may not be detected by IGF1 analysis.

Excretion of E1G was significantly lower in the follicular phase in ALL cycles. Early follicular serum oestradiol concentrations were also lower in ALL patients. As oestradiol production requires both LH and FSH (The European Recombinant LH study group., 1998), this may reflect a reduction in gonadotrophic stimulus that was detected in the reduced urinary LH excretion in ALL patients. There was also evidence for reduced steroid excretion during the luteal phase, particularly in cycles with short luteal phases. This reached statistical significance only for E1G excretion, reflecting the greater intercycle variability in P3G excretion.

Luteal insufficiency has been reported in the women with infertility and in female athletes. The role of luteal phase deficiency in causing infertility has been disputed because the diagnosis is not predictive of recurrence in subsequent cycles and studies have not revealed better outcome with progesterone treatment (Dawood, 1994). However, abnormalities in luteal phase have been detected in virtually all stimulation protocols used in vitro fertilization, partly due to decreased luteal LH secretion and insufficient corpus luteal function. Progesterone supplementation improves endometrial histology, and its necessity has been well established (Tavaniotou et al., 2001).

There is evidence for luteal insufficiency in female athletes. Exercising women with amenorrhoea exhibit a hypometabolic state. In female athletes with regular menses, a high proportion of cycles have been shown to be characterized by luteal phase deficiency with short luteal phase, reduced luteal progesterone excretion and reduced early follicular oestradiol excretion (De Souza et al., 1998, 2003). Disruption in LH pulsatility is seen as a primary factor in the high frequency of luteal phase defects in exercising women. These alterations may represent a metabolic adaption to an intermittent short-term negative energy balance.

Current fertility prospects for this group of survivors appear good, as evidenced by successful pregnancies in 3 of the 30 long-term survivors identified. However, this cohort is still young, with the majority not having tested their fertility. While none of the women showed evidence of overt ovarian damage, this may reflect our inability to detect this until relatively late when a woman has entered the perimenopause. The

risk of premature menopause following childhood chemotherapy remains to be clarified (Byrne et al., 1992). Ovarian dysfunction has been reported following chemotherapy for standard risk ALL, but the chemotherapy protocols included greater doses of alkylating agents (Quigley et al., 1989). The currents trends in treatment of ALL are to increase the intensity of chemotherapy to improve survival (Hann et al., 2000) .The late effects of the current treatment on reproductive function can only be measured many years after completion of therapy and the risk for the children currently undergoing chemotherapy without cranial irradiation may be greater than the cohort we are currently following whose chemotherapy schedules were less intense.

These data demonstrate that low dose cranial irradiation has an adverse effect on the hypothalamic – pituitary – ovarian axis that may be progressive over time. Apparently minor disturbances in LH secretion may have an effect on reproductive potential: conception in normal women is more likely in cycles with greater LH surges and higher luteal phase progesterone and oestradiol (Baird et al., 1999). Short luteal phases are associated with reduced fertility and early miscarriage (Soules et al., 1989). These data therefore indicate the importance of continuing assessment of reproductive function in this cohort of survivors to detect effects of treatment that may only become apparent may years later.

CHAPTER 5: Depletion of the ovarian reserve in young women following treatment for cancer in childhood: detection by anti-Müllerian hormone, inhibin B and ovarian ultrasound.

5.1 Introduction

Reproductive lifespan is determined by the number of primordial follicles and treatment that results in atresia of follicles will accelerate the menopause (Gougeon et al., 1994; Faddy et al., 1992; Byrne et al., 1992; Byrne et al., 1999; Tilly et al., 2002). Ovarian failure may occur immediately during or following treatment, or be delayed by a variable period (Whitehead et al., 1983; Wallace et al., 1989a; 1993). However for an individual, the risk cannot be readily predicted. With the arrival of more intense, multi agent protocols the risk of depletion of primordial follicles may increase.

The ability to predict a woman's reproductive lifespan would be of considerable value to these long-term survivors of childhood cancer who may be counselled not to delay childbearing. Current fertility prediction is limited. FSH is widely used in clinical practice but shows considerable inter cycle variability (Sherman et al., 1976; Ahmed Ebbiary et al., 1994; Wallach et al., 1995) and reflects the sum of both central hypothalamic drive and ovarian feedback. Direct products of the ovary, including inhibin B and anti-Müllerian hormone (AMH) have been investigated as markers of ovarian reserve (Seifer et al., 1997; Tinkanen et al., 1999; Creus et al., 2000; Dumesic et al., 2001); de Vet et al., 2002), the latter showing particular promise as a marker of the earliest growing follicles (Fanchin et al., 2003b). Biophysical measures including ovarian volume and antral follicle count (AFC) have also been shown to correlate with reproductive potential (Tomas et al., 1997; Syrop

et al., 1999; Scheffer et al., 1999; Scheffer et al., 2003; Yong et al., 2003). AFC has recently been demonstrated to be reduced in women who have survived childhood leukaemia (Larsen et al., 2003), but no hormonal effects were detected in that group.

The assessment of reproductive potential is complicated in those women taking the COCP. To evaluate ovarian function it has been necessary to stop the COCP, which is inconvenient and impractical in young women relying on this method of contraception. While it is likely that assessment of ovarian function during COCP administration will remain less accurate than in women cycling spontaneously, it would be of value to assess markers of ovarian function under these conditions.

The objective of this study was to investigate basal and stimulated hormone production by the ovary in women who have survived cancer treatment as children to detect and assess the degree of loss of the ovarian reserve. The relative value of endocrine markers and ultrasound investigation was compared, both in women with regular menstrual cycles and in those taking the COCP.

5.2 Methods

5.2.1 Subjects

We recruited two groups of women from the long term oncology follow up clinic at The Royal Hospital for Sick Children Edinburgh, and 2 groups of controls. Women were suitable for recruitment into the study if they were older than 16 years, more than 2 years since completion of therapy, had regular menstrual cycles (25 – 35 days) or a history of return of menses post chemotherapy and were currently taking the COCP (all containing 30μg ethinyl oestradiol). Women were excluded if they were thought to have premature ovarian failure, defined by irregular or absent menses and elevated gonadotrophins and currently on hormone replacement therapy, if they had not received any chemotherapy or radiotherapy for their primary diagnosis or they were thought not competent to give fully informed consent. Women without a history of childhood cancer and with a history of regular menses were recruited as controls. They were recruited by two methods: a) a poster campaign requested women who had regular menses, were not on the OCP and aged 18 – 30 years and were interested in taking part in a research study to contact the study coordinator and b) by a special study module student (a research module during 4th year of undergraduate training) who approached friends who were also undergraduates, regarding participation in a research study. Women were suitable for recruitment into the non-OCP group if they had not taken the OCP in the preceding 3 months. All

participants gave written informed consent. Full ethical approval was given by the local research ethics committee.

5.2.2.Study design

Women with spontaneous menstrual cycles attended during the early follicular phase (day 3 to 5), and those taking the COCP during the third week of their pill cycle i.e. between days 14 and 20. Venesection was performed and serum stored at –20°C for subsequent assay. Transvaginal ultrasonography was used to determine ovarian volume and the number of small antral follicles. All women were then administered an injection of 225 IU recombinant FSH (rhFSH, Gonal F; Serono, Welwyn Garden City, UK) subcutaneously, and returned 24 hours later for a further blood sample. Patients taking the COCP had a further blood sample on day 7 of a subsequent pill-free week for measurement of FSH to exclude occult ovarian failure: this was in the normal range (<10IU/L) in all women.

5.2.3 Outcome measures

Immunoassays for FSH, E2, inhibin A, inhibin B and pro α-C inhibin forms and ultrasound examinations were carried out as previously described (Yong et al., 2003). All ultrasound all examinations were performed by the same investigator using the same equipment (7Mhz probe, Toshiba Eccocee, Stirling, UK). AMH was

measured in a single assay (AMH ELISA, Beckman Coulter, High Wycombe, UK), intra assay CV 7%. Ovarian volume was calculated from 3 orthogonal diameters using formula for a prolate ellipsoid ($\pi/6$ x d1 x d2 x d3) and mean volume of the two ovaries calculated. AFC was determined as the mean of the number of follicles 2- 10 mm diameter in the two ovaries.

5.2.4 Statistical analysis

The day of onset of menses was defined as day 1 of the cycle. Hormonal data are presented as mean and SEM, and were compared by Student's t test after log transformation to correct for heterogeneity of variance. Ovarian volume and AFC were compared using the Mann-Whitney U-test.

5.3 Results

5.3.1 Subjects

We recruited 10 women aged 24 (16 – 34) years [mean (range)] with regular menstrual cycles (25-33 days) treated age 8 (3 – 12) years for cancer (table 4.1) and 11 controls(table 5.2) aged 23 (17-29) with regular menstrual cycles. We also recruited 10 women aged 20 (17-29) years on the COCP treated age 8 (4-15) years for cancer (table 5.3) and 10 controls aged 23 (22-26) years on the COCP (table 5.2). The diagnosis and treatment schedules were as detailed in tables 5.1 and 5.3. All women completed the study. There were no significant differences between age at investigation or body mass index between the patient groups and between patients and controls (data not shown). FSH administration resulted in a significant rise in serum FSH concentrations at 24 hr in all four groups ($P<0.01$).

5.3.2 Women with regular menstrual cycles

The biochemical data of cancer survivors at baseline are shown in table 5.4 and 24 hours post injection of FSH in table 5.5. The biochemical data of the controls at baseline are shown in table 5.6 and 24 hours post injection of FSH in table 5.7. Among women with regular spontaneous menstrual cycles, cancer survivors had significantly higher early follicular phase FSH compared to controls (7.5±1.4 vs

Table 5.1 Clinical information on diagnosis and treatment of patients with regular menstrual cycles not on the OCP

Pts	Age at diagnosis	Age at assessment	Diagnosis	Treatment
1	4	34	ALL	Chemo + CRT
2	11	16	Ewing's sarcoma	Chemo + groin RT
3	12	29	ALL	Chemo + CRT
4	11	27	Medulloblastoma	Craniospinal RT
5	5	17	ALL	Chemo + CRT
6	11	23	Rhabdomyosarcoma	Chemo
7	2	20	ALL	Chemo + CRT
8	8	24	Wilms tumour	Chemo
9	12	18	Rhabdomyosarcoma	Chemo + RT to face
10	4	18	NHL Stage 2	Chemo

Table 5.2 Details of controls, women with regular menses not on OCP and women on OCP

Subjects not on OCP	Age at investigation	Subjects on OCP	Age at investigation
1	17	1	22
2	29	2	22
3	24	3	24
4	23	4	23
5	21	5	22
6	25	6	23
7	22	7	23
8	21	8	26
9	21	9	22
10	26	10	22
11	28		

Table 5.3 Clinical information on diagnosis and treatment of patients on COCP

Pts	Age at diagnosis	Age at assessment	Diagnosis	Treatment
1	15	19	NHL stage 4	Chemo
2	5	21	ALL	Chemo + CRT
3	3	17	ALL	Chemo + CRT
4	2	17	ALL	Chemo + CRT
5	5	16	Hodgkin's	Chemo
6	9	29	Osteosarcoma	Chemo
7	11	18	Hodgkin's disease	Chemo
8	2	20	ALL	Chemo + CRT
9	1	20	Wilms tumour stage 2	Chemo
10	9	28	ALL	Chemo + CRT

Table 5.4 Biochemical data of patients not on OCP at baseline

Pt no	FSH U/L	AMH pmol/l	Inh B pg/ml	Inh A pg/ml	Pro αC pg/ml	Oestradiol pmol/l
1	5.7	13.5	69.3	2.8	46.2	169
2	10.2	2.7	47.4	2.8	58	195
3	4	11.4	143.6	14.4	331	155
4	18.5	0.4	53.2	1.9	92.6	134
5	4.3	21.1	117.1	8.2	209.9	138
6	6.1	1.4	135.7	11.2	196.4	136
7	6.7	15.2	107.9	6.5	127.2	267
8	3	31.2	114.9	6.3	344.4	175
9	10.1	17.2	116.5	9.2	124.7	325
10	6.7	16.2	75.4	9	90.5	292

Table 5.5 Biochemical data of patients not on OCP 24 hrs post injection of FSH

Pt no	FSH U/L	AMH pmol/l	Inh B pg/ml	Inh A pg/ml	Pro αC pg/ml	Oestradiol pmol/l
1	7.5	14.1	157	15.3	87.3	331.8
2	10.9	2.8	87.5	13.2	102.1	194.1
3	5.6	11.4	392.8	53.1	402.8	407
4	20.3	0.4	79.6	5	121.9	187.2
5	9.6	21.4	231.8	22.2	295.6	492.5
6	14.9	1.5	216.2	28.9	239.9	530.3
7	7.4	15.5	275.6	20.9	200	484.1
8	4.9	31.4	164	14.8	299.7	349
9	12.6	19	220.1	22.4	223.6	617.7
10	7.2	19.4	216.6	26.6	219.3	314.4

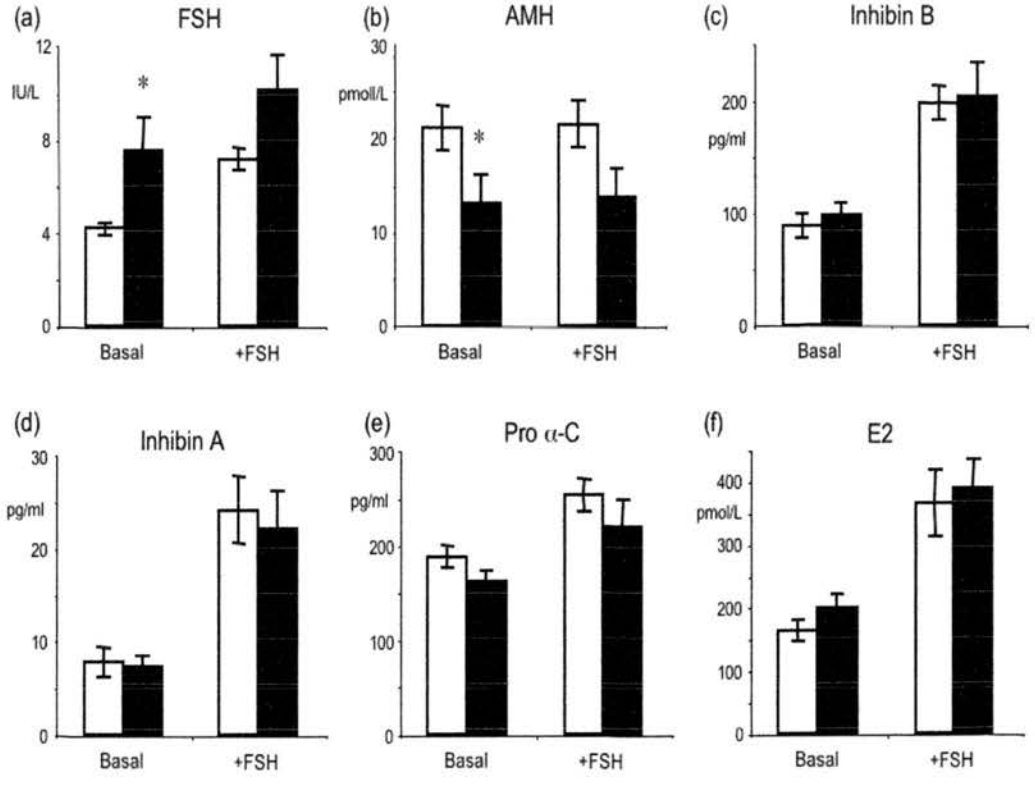
Table 5.6 Biochemical data of controls not on OCP at baseline

Pt no	FSH U/L	AMH pmol/l	Inh B pg/ml	Inh A pg/ml	Pro αC pg/ml	Oestradiol pmol/l
1	4.1	15.2	114	13.7	188.6	203.7
2	4.1	27.1	93.4	10.2	149	151.9
3	4.2	24.3	154.1	17.5	230.6	229.4
4	5.6	16	83.6	2.7	231.1	102.4
5	4.1	14.8	39.8	1.9	153.4	167
6	5.1	32.1	106.4	13.7	157.8	272.3
7	3.8	33.6	99.2	7.9	186.2	157.8
8	4.8	18.4	80.7	1.9	186.4	110.8
9	2.4	24.3	80.3	4.7	241.2	109.7
10	3.3	17.7		6.9	124.8	146.4
11	4.5	7.5	32.4	4.3	218.8	145.3

Table 5.7 Biochemical data of controls not on COCP 24 hrs post injection of FSH

Pt no	FSH U/L	AMH pmol/l	Inh B pg/ml	Inh A pg/ml	Pro αC pg/ml	Oestradiol pmol/l
1	7.4	13.4	218.9	36.3	276.5	322.2
2	9	29	209.2	30.6	222.3	466.1
3	7.7	19	297.7	43.9	257.9	796
4	8.2	18.1	136.2	12	251.3	164.4
5	5.1	16.4	198.6	11.2	175.8	201.1
6	6.4	30.1	188.8	39.1	297.9	4471.6
7	6.5	37.1	217.1	22.1	255.4	358.6
8	7.7	22.6	177.5	9.3	252.1	198.2
9	4.7	24.6	254.8	20	348.3	412.5
10	6.2	18.8	139.6	18.7	149.7	297.6
11	9.8	7.3	134.9	23	295.7	325.5

Figure 5.1 Serum FSH, AMH, inhibin A, B and proα-C and oestradiol in women with spontaneous menstrual cycles. Controls (open bars, n=11) and survivors of childhood cancer (filled bars, n=10). Blood samples were taken in the early follicular phase (basal) and 24 hr after administration of 225IU rhFSH (+FSH). Mean ± SEM. * P <0.05 vs controls.



4.2±0.3 IU/L, p = 0.02, figure 5.1a). AMH was significantly lower in cancer survivors than controls (13.0±3.0 vs 21.0±3.4pmol/L, P <0.05, figure 5.1b) and was unchanged following administration of FSH. There was no significant difference in early follicular phase inhibin A, B, proα-C or oestradiol between cancer survivors and controls, and all four hormones showed an increase in response to administration of rhFSH (figure 5.1 c-f, p<0.01 in each case). However post-FSH concentrations were also similar in cancer survivors and controls and there was no difference in increment in inhibin B from baseline to stimulated response between the two groups.

USS data for the cancer survivors are shown in table 5.8 and for the controls in 5.9. Ovarian volume was significantly smaller in cancer survivors than controls (3.0±0.5 vs 5.0±0.8 ml, P < 0.05, figure 5.2a). AFC was not significantly different between patients and controls (8.4±1.4 vs 9.0±1.2, figure 5.2b).

5.3.3 Women taking COCP

The biochemical data of cancer survivors at baseline are shown in table 5.10 and 24 hours post injection of FSH in table 5.11. The biochemical data of the controls at baseline are shown in table 5.12 and 24 hours post injection of FSH in table 5.13. Basal FSH, oestradiol and inhibins were, as anticipated, low in both cancer survivors and controls in the COCP groups (figure 5.3) with no significant differences in any of the hormones between cancer survivors and controls. Inhibin A and B were undetectable or very close to the limit of detection in both cancer survivors and controls. Inhibin B showed a small increase which was significant (p=0.003) in the

Table 5.8 USS data of patients not on COCP

Pt	Ovarian volume mls	Antral follicle count
1	1.8	4
2	6.3	5
3	3.7	8.5
4	0.5	3
5	2.8	13
6	2.1	4
7	4.5	10
8	2.5	9
9	3.2	12
10	2.7	8

Table 5.9 USS data of controls not on COCP

Pt	Ovarian volume mls	Antral follicle count
1	9.7	10
2	5.2	9.5
3	3	9
4	1.9	6
5	1.8	4
6	4.7	7
7	5.5	14.5
8	6.4	9.5
9	9	16
10	4.4	4.5
11	3.4	4

Figure 5.2 Average ovarian volume and antral follicle count in controls (open bars) and survivors of childhood cancer (filled bars), in women with spontaneous regular menstrual cycles and during COCP administration. Mean ± SEM. * P <0.05 vs control group, † P<0.02 vs women with spontaneous cycles.

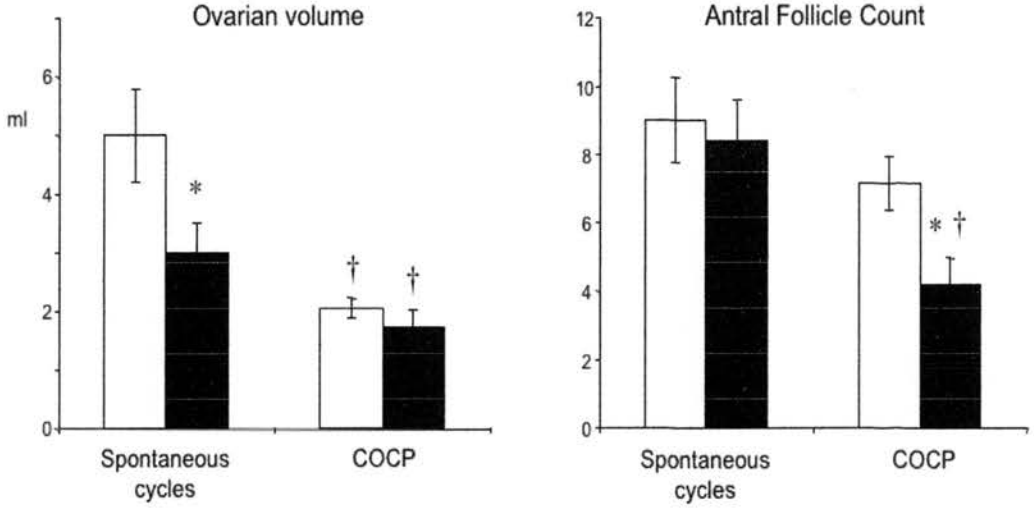


Table 5.10 Biochemical data of patients on COCP at baseline

Pt no	FSH U/L	AMH pmol/l	Inh B pg/ml	Inh A pg/ml	Pro αC pg/ml	Oestradiol pmol/l
1	0.16	2.5	7.8	1.9		89.5
2	3.3	2.5	7.8	1.9	66.2	87.7
3	1.22	9.2	7.8	1.9	3.1	76.7
4	0.2	17.7	7.8	1.9	50.8	54.3
5	0.2	0.4	7.8	1.9	124	55.8
6	0.8	4.3	7.8	1.9	3.1	94
7		0.4				
8	2.5	17.7	7.8	1.9	50.4	71.2
9	0.5		25.1	2.98	158.4	
10	0.5	28.7	7.8	1.9	44.3	110.1

Table 5.11 Biochemical data of patients on COCP 24 hrs post injection of FSH

Pt no	FSH U/L	AMH pmol/l	Inh B pg/ml	Inh A pg/ml	Pro αC pg/ml	Oestradiol pmol/l
1	4.2	4.9	7.8	1.9		89.8
2	8.1	17.7	125	4.6	71.6	78.5
3	5.6	9.9	7.8	1.9	134.4	271.6
4	5.1	17.1	49.8	1.9	75.4	68.3
5	5.1	0.4	7.8	1.9	77.7	66.1
6	3.3	6	7.8	1.9	75.4	103.9
7						
8	4	21.5	72.6	1.9	112	98.4
9	4.4		193.8	15.1	218.7	141.7
10	1.9	28.6	7.8	1.9	3.1	132.5

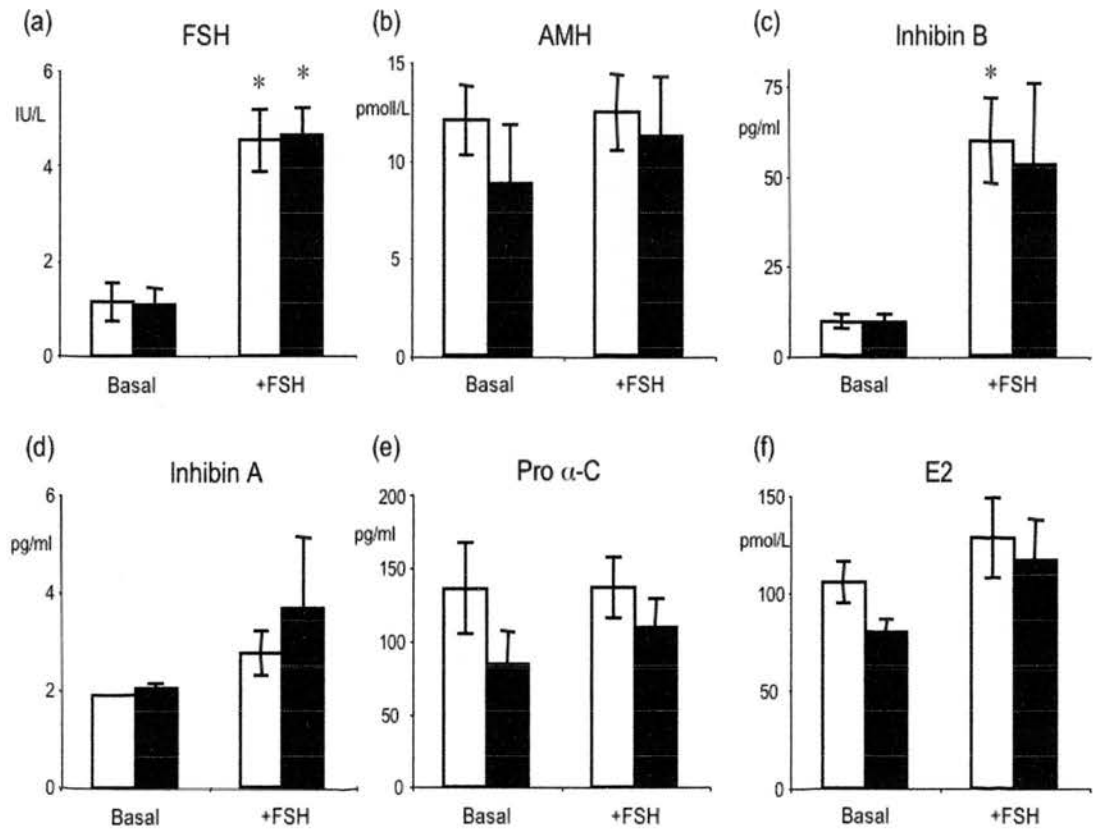
Table 5.12 Biochemical data of controls on COCP at baseline

Pt no	FSH U/L	AMH pmol/l	Inh B pg/ml	Inh A pg/ml	Pro αC pg/ml	Oestradiol pmol/l
1	2.3	9	7.8	1.9	89	121.8
2	0.1	12.5	7.8	1.9	72.7	139.8
3	2.7	22	7.8	1.9	171	97.3
4	0.5	5.5	7.8	1.9	336.6	79.6
5	0.1	21.3	7.8	1.9	286.9	178
6	0.3	7.2	7.8	1.9	75.2	65.7
7	0.3	8	7.8	1.9	105	87.3
8	0.6	12.2	27.3	1.9	51.5	97.3
9	0.6	10.2	7.8	1.9	77.4	76
10	3.7	12.2	7.8	1.9	94.1	111.9

Table 5.13 Biochemical data of controls on COCP 24 hrs post injection of FSH

Pt no	FSH U/L	AMH pmol/l	Inh B pg/ml	Inh A pg/ml	Pro αC pg/ml	Oestradiol pmol/l
1	5.7	9.2	92.6	3.7	160.1	235.6
2	4.3	9.2	28.4	1.9	128.1	101.3
3	5.8	23.6	37.6	1.9	80.6	139.8
4	3.3	5.4	35.2	1.9	182.5	85.5
5	2.3	22.6	132.2	4.5	233.6	198.9
6	3.3	9.1	33.3	1.9	75.9	78.9
7	3.1	8.9	84.1	1.9	101.6	62.4
8	3.6	13.4	33.6	1.9	79.6	78.9
9	4.5	10	28.2	1.9	77.4	88.8
10	9.4	13	93.9	5.9	243.9	216.9

Figure 5.3 Serum FSH, AMH, inhibin A, B and proα-C and oestradiol in women on COCP. Controls (open bars, n=10) and survivors of childhood cancer (filled bars, n=10). Blood samples were taken in the third week of a COCP cycle before (basal) and 24 hr after administration of 225IU rhFSH (+FSH). Mean ± SEM. * P <0.05 vs basal.



control group but not in the cancer survivor group. However analysis of individual inhibin B responses identified six cancer survivors with undetectable inhibin B pre-FSH (< 7.8 pg/ml) in whom inhibin B remained undetectable after FSH stimulation whereas control women, who also had undetectable inhibin B concentrations pre-FSH, all showed a rise of greater than 20 pg/ml in response to FSH. Inhibin A remained undetectable in all except three controls and two cancer survivors who showed a response, and proα-C and AMH were unchanged. Serum oestradiol showed a small but not statistically significant increase in response to FSH administration in both groups, with no differences between groups.

USS data for the cancer survivors are shown in table 5.14 and the controls table 5.15. Ovarian volumes were significantly lower in both COCP cancer survivors and controls compared to the groups with spontaneous ovarian activity (1.7±0.3 vs 3.0±0.5 ml, P<0.05 and 2.1±0.2 vs 5.0±0.8 ml, p=0.002 respectively, figure 5.2a). There were no significant differences in ovarian volume between COCP-taking patients and controls. AFC however was significantly lower in COCP-taking cancer survivors than controls (4.2±0.8 vs 7.2±0.8, P = 0.02, figure 5.2b). COCP-taking cancer survivors who showed no inhibin B response to FSH had slightly lower AFC than those who did respond (3.6 vs 4.6) but these differences did not reach statistical significance.

Table 5.14 USS data of patients on COCP

Pt	Ovarian volume mls	Antral follicle count
1	1.3	3
2	1.4	8
3	2.8	5
4	0.7	1
5	1	2
6	0.9	3
7	1.4	5
8	2	3
9	3.3	7
10	1.7	2

Table 5.15 USS data of controls on COCP

Pt	Ovarian volume mls	Antral follicle count
1	2	8.5
2	1.6	7.5
3	2.4	8
4	1.2	3
5	2.1	6
6	2.7	5
7	1.9	8
8	3	8
9	1.9	5.5
10	1.7	12

5.4 Discussion

Fertility is a major concern among women who have survived cancer during childhood. Some women may develop an early menopause, but others may progress through puberty normally and have regular menstrual cycles with normal endocrine profiles. As the agents used to treat the malignancies of childhood will destroy a greater or lesser number of ovarian primordial follicles, it is of value to be able to assess accurately the effect of treatment on the ovarian reserve even in those women with apparently normal ovarian function. This has proved difficult because of the low metabolic activity of primordial follicles, and the number of growing follicles, particularly the FSH-dependent small antral follicles, has been widely used as a substitute as this number is believed to reflect the number of primordial follicles (Gougeon et al., 1994). Early data from autopsy specimens of children with leukaemia directly demonstrated a reduction in antral follicle number following chemotherapy (Himelstein-Braw et al., 1978). In this study we have demonstrated both hormonal and biophysical evidence of partial loss of the ovarian reserve in young cancer survivors with regular cycles.

Two hormonal differences were detected between cancer survivors and controls. Firstly, early follicular FSH was significantly elevated, although was high in only one patient (18.5 IU/L, others all <10.2 IU/L). However there was a striking fall in serum AMH concentrations, while the other direct products of the ovary, oestradiol and the inhibins A, B and pro-αC, were unchanged. AMH is produced by the

granulosa cells of small growing follicles (Baarends et al., 1995) and is involved in the regulation of primordial follicle recruitment (Durlinger et al., 1999). It has recently been suggested to be a marker of ovarian ageing (de Vet et al., 2002). The circulating concentration shows little fluctuation over the menstrual cycle (Cook et al., 2000) but is a relatively good predictor of the number of small FSH-sensitive follicles and thus of the number of oocytes recovered following controlled ovarian stimulation (van Rooij et al., 2002; Seifer et al., 2002; Fanchin et al., 2003b). Consistent with previous reports, serum AMH was unchanged by administration of a single dose of FSH. The present data are thus consistent with a partial depletion of the follicular reserve in these patients and is the first demonstration of a fall in an ovarian hormone in such cancer survivors with regular menstrual cycles.

Ovarian volume but not AFC was also reduced in the cancer survivors compared to controls. Both ovarian volume and AFC are indirect markers of the ovarian reserve (Tomas et al., 1997; Syrop et al., 1999; Scheffer et al, 1999, 2003; Yong et al., 2003), and have been reported to be reduced in female survivors of childhood cancer (Larsen et al., 2003). Inhibin B, a product of these follicles, was also normal. It has recently been demonstrated that the value of inhibin B as a measure of the ovarian reserve is greatly increased following administration of a single dose of FSH to stimulate granulosa cell function in small healthy follicles (Yong et al., 2003). While these results show the expected increase in inhibin B following FSH administration, there remained no difference between patients and controls. Together with the AFC results, these data are consistent with these cancer survivors having a near-normal compliment of small antral follicles. The discrepancy between these normal results

and the clearly reduced AMH data may illustrate the problems inherent in trying to assess the number of primordial follicles present using only indirect means. As AMH is the product of the smallest growing follicles, it may more accurately reflect the ovarian reserve than AFC and basal and stimulated inhibin B. The latter two markers reflect the number of FSH-sensitive small antral follicles and therefore show relationships to the number of oocytes recovered following controlled ovarian stimulation, but it appears they are unable to reflect the reduced ovarian reserve exhibited by the cancer survivors investigated here.

Groups of cancer survivors and controls taking the COCP were also investigated in this study. The ability to carry out accurate assessment of the ovarian reserve under such conditions would be of great practical value in the light of the high prevalence of oral contraceptive use among young women. Assessment under hypogonadotrophic conditions might also be advantageous: GnRH analogue-induced hypogonadotrophism with subsequent FSH stimulation has been shown to greatly increase the correlation between inhibin B and oocyte recovery following controlled ovarian stimulation (Yong et al., 2003). As with the spontaneously cycling groups, cancer survivors taking the COCP showed both hormonal and biophysical differences from the control group although the differences were in distinct measures of ovarian function. During COCP administration, inhibin B was suppressed to undetectable concentrations in both cancer survivors and controls. In response to FSH administration, the two groups showed similar responses overall, but analysis of individual responses showed that whereas all controls showed a response to FSH, only 6/10 cancer survivors showed a response, inhibin B remaining undetectable in

the others. AFC was lower in women taking the COCP (significantly so for the cancer survivor groups), but there was also a significant difference in AFC between COCP-taking cancer survivors and COCP-taking controls. Thus, in contrast to the spontaneously cycling groups, during COCP administration there were differences between cancer survivors and controls in AFC and inhibin B. These results again suggest that cancer survivors show a degree of depletion of the ovarian reserve, and under conditions of hypogonadotrophism, this is reflected in the number of FSH-sensitive antral follicles. As the degree of loss of the ovarian reserve is modest, it may be that under normal conditions the larger number of growing follicles has obscured our ability to detect differences between the groups.

Ovarian volume and serum AMH were however not significantly different between COCP-taking cancer survivors and controls. Both these markers were significantly reduced in the COCP controls compared to the spontaneously cycling controls, indeed to values similar to those found in the spontaneously cycling cancer survivors. It appears likely that the effect of the COCP has obscured the value of these markers of the ovarian reserve. Ovarian volume, AFC, inhibin B and AMH are all to greater or lesser extents markers of the ovarian reserve. They are however both indirect and while inter-related reflect slightly different aspects of ovarian function. The present results further illustrate the relationships between them under different conditions, and confirm the need for a range of markers for a full assessment of the ovarian reserve.

Some chemotherapy agents are known to be more gonadotoxic than others (Mackie et al, 1996; Whitehead et al., 1983). In particular, alkylating agents are recognised to carry a significant risk of premature ovarian failure. Greater than half the group of cancer survivors (ALL and Wilms' tumour survivors) have received treatment believed to have little or no ovarian toxicity (Wallace et al., 1993) although craniospinal irradiation may have indirect adverse effects on ovarian function (Bath et al., 2001). However, of the six patients taking the COCP who showed no inhibin B response to FSH, three had been treated for ALL. Analysis is limited by the small number of patients treated for a variety of primary diagnoses with different chemotherapy regimens, but these data show even these therapies are associated with a detectable loss of ovarian reserve.

In conclusion, survivors of childhood cancer were demonstrated to have suffered a depletion of the ovarian reserve despite maintaining regular menstrual cycles. Serum AMH concentrations and measurement of ovarian volume by transvaginal ultrasound scan were found to be the clearest indicators of this. Evidence of a similar effect was also detected despite COCP administration using different markers. Regular menstrual cycles and normal early follicular phase FSH do not therefore confirm the absence of damage to the ovary, and such patients should be advised accordingly. Long term follow up of these patients is necessary to further evaluate ovarian function and definitively correlate these indirect markers of the ovarian reserve investigated here with true reproductive lifespan. Prospective systematic collection of data will also, in the future, allow a more accurate prediction of reproductive potential.

CHAPTER 6: Ovarian and uterine Characteristics after total body irradiation in childhood and adolescence: Response to sex steroid replacement

6.1 Introduction

The majority of children treated for acute leukaemia are cured with intensive chemotherapy and will be fertile (Nicholson et al., 1993; Wallace et al., 1991; Wallace et al., 1993). For patients with high risk or relapsed disease the prospect of cure has been improved with the increasing success of BMT with TBI as the conditioning treatment (Chessels., 1998).

The aim of this study was to investigate ovarian function and uterine characteristics in long term survivors following TBI. Uterine size, blood flow and endometrial thickness were determined by ultrasonography in women with premature ovarian failure in response to exogenous sex steroid replacement and in response to endogenous sex steroid production in women with spontaneous ovarian function. The events of implantation and placentation will reflect prevailing endometrial function which can be further evaluated by endometrial biopsy.

6.2 Methods.

6.2.1 Subjects

Eight post pubertal women who had been treated with total body irradiation as children/ adolescents were studied (Group A). They were recruited from a total of nine women treated with TBI in paediatric centres in Scotland in long term remission. The one patient not recruited was receiving treatment for Hepatitis C. They were all treated with a total of 14.4 Gray in 8 fractions over 3 days, in first or second remission for leukaemia. The chemotherapy protocols were the MRC ALL or AML trial according to the time of primary diagnosis. Those in second remission had all previously received cranial irradiation. Of the 8 patients in group A, 5 were available for further study. Two comparison groups were studied; groups B and C. Women in group B (n = 12) were treated with chemotherapy and cranial irradiation and were in first remission following treatment with standard UK protocols for acute leukaemia (Burnett et al., 1997). They were recruited from the late effects clinic at the Royal Hospital for Sick Children, Edinburgh. Group C (n = 5) were healthy controls with regular menstrual cycles and no history of childhood malignancy. They were recruited following a poster campaign to recruit healthy young women with regular menstrual cycles, not on the OCP. The age at treatment and age at assessment for each group are shown in table 6.1.

Table 6.1. Age at treatment and age at assessment for each group [median(range)]

	age at treatment	age at assessment
Group A (n=8)	11.5 (5.9 - 15.1) years	17.1 (15.4 - 21.5) years
Group B (n=12)	6.7 (3.8 - 13.5) years	21.8 (15.8 - 32.8) years
Group C (n=5)		25.2 (24.1 - 27.1) years

Table 6.2. Details of physiological sex steroid replacement regimen.

	Week 1	Week 2	Week 3	Week 4
Oestradiol	100 mcg/24 hrs	150 mcg/24 hrs	150 mcg/24 hrs	150 mcg/24 hrs
Progesterone			200 mg/12 hrs	200 mg/12 hrs

6.2.2 Study design

Each woman had an initial assessment to determine pubertal development, date of menarche, regularity of menses and, where appropriate, age at commencement of sex steroid replacement. Spontaneous ovarian function was assessed in women taking sex steroid replacement (SSR) after discontinuing that treatment for 4 weeks. Ovarian failure was confirmed by raised serum gonadotrophins, low serum oestradiol and absent menses.

Subjects with ovarian failure then commenced a physiological regimen of sex steroid replacement administered as transdermal oestradiol patches (Estraderm TTS, Novartis, Horsham, UK) and vaginal progesterone pessaries (Cyclogest, Shire Pharmaceuticals Ltd, Andover, UK) as detailed in table 6.2. This regimen has previously been shown to produce physiological levels of sex steroid replacement in women with premature ovarian failure (Critchley et al., 1990). The pSSR regimen was continued for 3 cycles, and detailed assessment was undertaken during the third cycle. Alternate day urine samples were collected which were stored at -20 ^{o}C until assayed for LH, oestrone and pregnandiol. Subjects were assessed at times in the cycle equivalent to early follicular (day 3 - 5), mid-late follicular (day 9 - 12) and mid luteal phase (day 22 - 24) by ultrasound scan and hormone measurements. In sexually active women consent was sought for collection of an endometrial biopsy in the simulated mid-luteal phase.

In women with spontaneous cycles, ovarian function was assessed for 3 cycles by collection of a daily urine sample. Measurement of LH was used to accurately time the mid luteal assessment and oestrone and pregnandiol were measured to confirm ovulation. Subjects underwent detailed assessment of ovarian and uterine function during the third cycle as described for women on pSSR at early follicular, mid follicular and mid luteal stages of the cycle.

Ethical approval was given by the local ethics committee and informed consent was obtained from all women in the study.

6.2.3 Ultrasound scan

All ultrasound examinations were performed by one of two consultant radiologists trained in gynaecological scanning using a Hitachi EUB 555. Scans were performed transvaginally (6.5 MHz transducer) in sexually active women and transabdominally (3.5 MHz transducer) in those who were not. The measurements recorded were uterine volume, blood supply and endometrial thickness and ovarian volume and the presence and size of follicles. Uterine volume was calculated from the three dimensions, length (d_1), measured from the fundus to the external os, the transverse diameter (d_2) and the antero-posterior diameter (d_3) and by assuming the forms to be ellipsoid, using the formula for a prolate ellipsoid ($d_1 x d_2 x d_3$) x 0.523. The endometrial thickness was measured on a sagittal section, the maximum antero-posterior diameter was measured for both layers. Uterine artery blood flow was

evaluated using colour and pulsed flow doppler. The PI was used to express blood flow impedance distal to the point of sampling. The PI was derived from the flow velocity wave form, according to the formula PI = A - B/mean, where A is peak systolic Doppler shift frequency, B is the end diastolic shift frequency and the mean is the mean maximum Doppler shift frequency over the cardiac cycle (Taylor et al., 1985; Steer et al., 1990). The ovarian volume was measured in three orthogonal diameters and the same formula for a prolate ellipsoid applied.

6.2.4 Endometrial morphology

In sexually active women an endometrial biopsy was collected in the mid luteal phase (natural or simulated). The samples were conducted with a Pipelle suction curette (Laboratoire CCD, Paris, France) and endometrium placed in neutral buffered formalin prior to embedding in paraffin. Subsequently 5 micron sections were cut from the paraffin blocks for histological examination and immunohistochemical detection of both ER and PgR (Critchley et al., 1998; Snijders et al., 1992)

6.2.5 Hormone analyses

Urinary oestrone and pregnandiol were measured using an "in house" ELISA using HRP-conjugate as label and solid-phase second antibody separation (for oestrone CV < 4%; for pregnandiol CV <13%). Urinary LH was measured by a two-site IRMA

(Serono MAIA clone) (CV < 12%). Urinary hormone concentrations were corrected for creatinine concentration. Serum oestradiol was measured by competitive immunoassay using the Boehringer Mannheim Elecsys (Mannheim, Germany). Progesterone, FSH and LH were measured by microparticle enzyme immunoassay on the Abbott Axsym (Chicago, Illinois). Inhibin B was measured using a two-site ELISA using plates coated with specific monoclonal antibodies to the ßB subunit of inhibin B as previously described (Groome et al., 1996). Assay sensitivity was 7.8pg/ml, intra- and inter-plate CVs were 10.6% and 11.4% respectively.

6.2.6 Statistical analysis

The ultrasound and serum assay results were not normally distributed and therefore non parametric tests was used to analyse the data. The data are therefore presented as medians, ranges and interquartile ranges. For within group comparisons we used the Wilcoxon signed ranks test. For between group comparisons we used the Wilcoxon rank sum test. To investigate the relationship between non parametric variables we used Spearman's rank correlation coefficient.

6.3 Results

6.3.1 Subjects

Six of the 8 women in group A (treated with TBI) had absent ovarian function requiring sex steroid replacement and all had required sex steroid treatment for pubertal induction. Two patients treated pre pubertally had progressed spontaneously through puberty without SSR, although their cycles were irregular. The timing of treatment according to pubertal stage is as shown in table 6.3. Of this group, 4 with ovarian failure and 1 with spontaneous menses underwent further detailed investigation.

All 12 women in group B (table 6.4) and all controls (group C) had progressed spontaneously through puberty and had regular menses. There was no significant difference between these 2 groups in ultrasound scan data, blood results or endometrial biopsy and their data were therefore combined as the comparison group.

Table 6.3. Patient details of group A.

Pt no	diagnosis	age at TBI	breast stage at TBI	age at assessment	ovarian function	full study data
1	ALL	15.1	St V	19	F	N
2	CML	12.7	St V	21.5	F	Y
3	AML	12.5	St V	16.7	F	Y
4	ALL	13.6	St IV	17.2	F	Y
5	ALL	10.5	St I	16.0	F	N
6	ALL	7.9	St I	15.5	NF	Y
7	ALL	5.9	St I	15.6	NF	N
8	ALL	4.9	St I	14.1	F	Y

Ovarian function: F, failed ; NF, not failed.

Participation in full study: Y, yes; N, no.

Table 6.4 Patient details of Group B

Patient	Diagnosis	Age at diagnosis	Age at assessment
1	ALL	3.2	16.3
2	ALL	3.9	30.1
3	ALL	1.9	22
4	ALL	5.5	17.3
5	ALL	2.1	19.5
6	ALL	11.2	32.6
7	ALL	3.2	16.8
8	ALL	6	23.2
9	ALL	10	17.3
10	ALL	7.2	23.7
11	ALL	2.2	16
12	ALL	13.1	24.3

6.3.2 Ovarian function

Four women with ovarian failure following TBI were assessed after discontinuing hormone replacement therapy 4 weeks previously. Gonadotrophins were elevated, oestradiol was low and inhibin B was undetectable confirming ovarian failure (tables 6.5 and 6.6). On USS ovarian structures were detectable in only 2 of the 4 patients (0.5, 0.7 mls), and were significantly smaller than those in Groups B and C [5.1 (2.48 - 8.48mls)] [median (range)]($p < 0.05$). During the third cycle of pSSR hormone concentrations were determined on days 3-5 (table 6.7) and 22-24 (table 6.8). Gonadotrophins were greatly reduced towards or into the normal range, and oestradiol and progesterone concentrations were also normalised (table 6.5).

One patient (patient no 7) with preserved ovarian function but irregular menses following TBI showed moderately elevated gonadotrophins, in the follicular phase, FSH 26 U/L and LH 8.9 U/L, with undetectable inhibin B consistent with incipient ovarian failure. Following ovulation (confirmed on urine LH surge) serum gonadotrophins, oestradiol and progesterone were within the normal range for the mid luteal phase. The volume of each ovary was 0.8 and 0.6 mls.

For the 4 women with ovarian failure, urine hormone measurements during the third cycle confirmed that pSSR treatment resulted in urinary oestrone and pregnandiol concentrations not significantly different from the quoted reference range. In 2 of the 4 patients in group A with ovarian failure a mid cycle urinary LH surge was demonstrated indicating normal response of the hypothalamic-pituitary-gonadal axis.

Table 6.5 Biochemical data of Group A at baseline

Pt	FSH U/L	LH U/L	Oestradiol pmol/l	Inhibin B pg/ml
2	108	55	51	<7.8
3	79	67.5	<37	<7.8
4	88	47.7	28	<7.8
8	107	45.7	<37	<7.8

Table 6.6. Endocrinology of patients with ovarian failure (n=4) [median(range)].

	Baseline	Cycle 3,day 3 - 5	Cycle 3,day 22 - 24
FSH U/l	97 (88 - 108)	10 (5.3 - 10.9)	6.3 (3.8 - 10.9)
LH U/l	51 (45 - 67)	2.8 (1 - 4.1)	1.1 (1 - 1.3)
Oestradiol pmol/l	14 (0 - 51)	422 (136 - 1736)	407 (215 - 965)
Progesterone nmol/l	0	0	18.3 (12.6 - 29.4)
Inhibin B pg/ml	<7.8	<7.8	<7.8

Table 6.7 Biochemical data of group A during 3rd month of pSSR on day 3-5 or day 3-5 of spontaneous cycle (pt 6).

Pt	FSH U/L	LH U/L	Oestradiol pmol/l	Inhibin B pg/ml
2	9.9	2	433	<7.8
3	10.3	4.1	417	<7.8
4	10.9	1.0	57	<7.8
6	26	8.9	38	
8	5.3	3.6	1736	<7.8

Table 6.8 Biochemical data of Group A during third month of pSSR on day 22-24 or day 22-24 of spontaneous cycle (pt 6)

Pt	FSH U/L	LH U/L	Oestradiol pmol/l	Progesterone
2	6.6	1.1	313	16.5
3	3.9	1.3	965	12.6
4	10.9	1.0	136	29.4
6	4	3	243	15.7
8	3.8	1	215	28.1

Women with spontaneous ovarian function were confirmed to be ovulating by the presence of an urinary LH surge followed by a rise in urinary pregnandiol.

6.3.3 Uterine characteristics post TBI

Four women in group A were confirmed to have ovarian failure after discontinuing their standard SSR for 4 weeks. Baseline assessment, 4 weeks after discontinuing their standard SSR, demonstrated uterine volume significantly reduced compared to Groups B and C ($p < 0.01$), undetectable uterine blood flow in 3 of the 4 women and no significant endometrial tissue (table 6.9). Following 3 cycles of pSSR they were reassessed (tables 6.10 and 6.11). There was an increase in uterine volume from 6.5 (1.7 - 12.7) ml [median (range)] at baseline to 16.5 (8.3 - 27.4) ml during the third cycle (table 6.12). The increase in uterine volume from baseline for each patient is shown in Fig. 6.1 and demonstrates that each patient had a sustained increase in uterine volume through the 3rd cycle of pSSR. Uterine volume measured during third cycle showed a significant correlation with age at TBI ($p< 0.05$) (fig 6.2). Endometrial thickness also increased from 0 (0 - 2.6) mm at baseline to 5.2 (3.5 - 7.5) mm during the mid luteal phase of the third cycle (table 6.12). Uterine artery blood flow, not detectable in 3 of the 4 patients at baseline, was detected in all patients during the third month of pSSR at which time uterine pulsatility index was within the normal range at 2.6 (1.9 - 3.4) (table 6.10).

Table 6.9 USS data of women in group A at baseline

Pt	Uterine volume mls	Pulsatility index	Endometrial thickness mm	Ovarian volume mls
2	12.7	3.24	2.6	0.5
3	10.2	Not seen	0	Not seen
4	1.7	Not seen	0	Not seen
8	2.8	Absent end diastolic flow	0	0.7

Table 6.10 USS data of women in group A during 3rd month of pSSR, day 3-5 or during spontaneous cycle day 3-5 (pt 6)

Pt	Uterine volume mls	Pulsatility index (left)	Endometrial thickness mm	Ovarian volume mls
2	17.4	2.64	5.4	Not seen
3	27.4	1.83	4	Not seen
4	15.5	2.73	0	Not seen
6	11.6	3.76	3	0.51
8	8.3	2.63	3	Not seen

Table 6.11 USS data of women in group A during 3rd month of pSSR, day 22-24 or during spontaneous cycle day 22-24 (pt 6)

Pt	Uterine volume mls	Pulsatility index (left)	Endometrial thickness mm	Ovarian volume mls
2	18.6	3.09	5.9	Not seen
3	25.9	3.22	5.5	Not seen
4	17.5	3.34	4.8	Not seen
6	12.4	1.46	8	1.1
8	7	2.64	3.5	Not seen

Table 6.12. Uterine volume and endometrial thickness of women with ovarian failure following treatment with TBI (n = 4) at baseline and after exposure to pSSR [median(range)].

	Baseline	3rd cycle of pSSR
Uterine volume (mls)	6.8 (1.7 - 12.7)	17.3 (7.0 - 25.9)
Endometrial thickness (mms) day 22-24	0 (0 - 2.6)	5.4 (3.5 - 7.5)

Figure 6.1 Change in uterine volume in patients in Group A from baseline compared to 3rd cycle of pSSR. Patients no 2, 3 and 4 were treated peri/post pubertally. Patient no 8 was treated pre pubertally. Pt no 2 = □ , Pt no 3 = O , Pt no 4 = ◇ ,Pt no 8 = ▲.

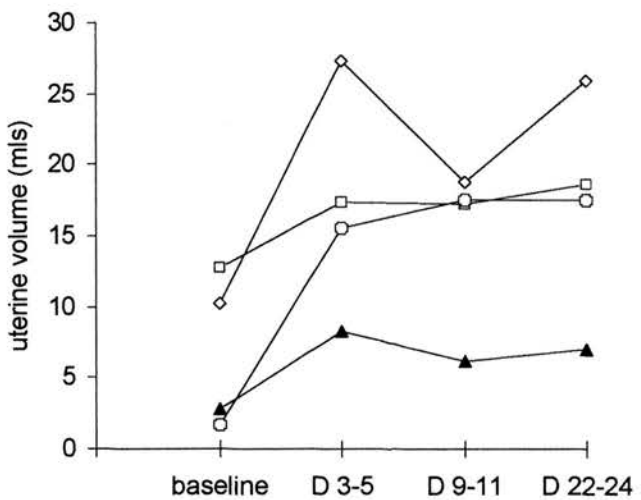


Figure 6.2 Correlation between uterine volume during 3rd cycle of pSSR (n = 4) or spontaneous ovarian function (n = 1) and age at irradiation ($p<0.05$).

6.3.4 Comparison of Groups A, B and C

The data from women in Group A following exposure to physiological sex steroid serum concentrations (pSSR for 3 cycles (n=4) or endogenous production (n=1, patient no 7)) were compared to that from women in Groups B (table 6.13) and C (table 6.14).

The uterine volume of women in group A [15.5 (8.3 - 27.4) ml] remained significantly smaller than in groups B and C [38.9 (26.8 - 64.0) ml] ($p< 0.01$) (table 6.15). Endometrial thickness increased through the cycle in all women studied and was not significantly different between the 2 groups. During the luteal phase the endometrial thickness in group A (table 6.11) was 5.5 (3.5 - 8) mm and in groups B (table 6.16) and C (table 6.17) 7.8 (6.6 - 12) mm (table 6.15). There was a significant correlation between urine oestrone concentrations and endometrial thickness in those on pSSR ($p < 0.05$). There was no significant difference in uterine artery pulsatility index during third cycle between group A [PI = 2.4 (1.9 - 3.4)] and groups B and C [PI = 2.1 (1.5 - 3.1)].

6.3.5 Endometrial morphology

An endometrial biopsy in the simulated mid luteal phase was obtained from patient no 2. Histological examination showed convoluted glands in an oedematous stroma with spiral arteriole formation. The histology and immunohistochemical distribution of oestrogen and progesterone receptors displayed features consistent with secretory

phase endometrium. In the early secretory phase ER immunoreactivity declined more markedly in the stromal cells, whereas ER expression in the glands decreased more gradually. Progesterone receptor expression declined in the glandular epithelium.

Table 6.13 USS data of women in group B day 3-5

	Uterine volume	Pulsatility index (left)	Endometrial thickness mm	Left Ovarian volume mls	Right Ovarian volume mls
1	40.6	3.14	4	1.54	3.4
2	35.5	2.04	1.4	2.02	3.1
3	64	1.67	7	3.26	7.5
4	33.8	2.39	4.5	4.55	3.1
5	57.9	2.19	3.6	8.6	3.3
6	46.8	2.59	6	8.8	5
7	38.9	2.51	1.4	7.7	9.2
8	26.8	2.77	7.6	3.9	3.4
9	26.9	2.23	1.3	2.7	Not seen
10	47.4	2.32	1.4	4.9	5.6
11	28.1	2.58	2.5	6.7	5.7
12	51.5	3.17	3.5	3.8	6.1

Table 6.14 USS data of women in Group C Day 3-5 of cycle

	Uterine volume	Pulsatility index (left)	Endometrial thickness mm	Left Ovarian volume mls	Right Ovarian volume mls
1	48.3	2.98	1.4	3.5	4.8
2	36.4	1.65	3	3.5	6.6
3	53.2	1.85	5.3	8	6
4	46.4	1.45	5.8	9.2	8.7
5	39.2	2.47	4.5	6.8	7.1

Table 6.15. Uterine volume and endometrial thickness of women in Group A (n = 5) during 3rd cycle compared to Groups B and C (n = 17). *p < 0.01; ** p > 0.05

	Group A	Groups B and C
uterine volume (mls)	16.3 (7.0 - 25.9)*	41.5 (28.1 - 57.9)
endometrial thickness (mm) day 22 - 24	5.9 (3.5 - 8.0)**	8.7 (6.6 - 12.4)

Table 6.16 USS data of women in group B day 22-24

	Uterine volume mls	Endometrial thickness mm
1	43.1	8
2	37.4	8
3	56.3	11.6
4	36.4	6.6
5	53.4	7.8
6	48.6	12.4
7	38.5	12
8	28.1	6.9
9	29.4	6.7
10	51.8	7.6
11	41.1	8.7
12	57.9	6.7

Table 6.17 USS data of women in Group C Day 22-24

	Uterine volume	Endometrial thickness mm
1	49.4	7.6
2	39.2	8.4
3	54.1	9.5
4	41.2	7.3
5	39.9	11.2

6.4 Discussion

Following TBI in childhood and adolescence we have confirmed that the majority of women have permanent ovarian failure (Ogilvey-Stuart et al., 1992; Sanders et al., 1996). This is however the first study to assess the uterine characteristics and the response to pSSR after TBI in young women. At baseline all these women had a small uterus, with poor blood flow and absent endometrium. Following physiological sex steroid replacement for 3 months all measures of uterine function improved such that there was no significant difference in uterine blood flow and endometrial thickness from the comparison group. Uterine volume, although increased with pSSR, remained significantly less than the comparison group.

TBI carries a significant risk of permanent ovarian failure. Although our numbers are small, the risk appears greatest for girls treated post pubertally, which is consistent with reports from other groups (Sarafouglou et al., 1997; Sanders et al., 1996; Sanders et al., 1986; Leiper et al., 1987). In woman treated pre pubertally with TBI ovarian function may be preserved but there remains a risk of early ovarian failure (Byrne et al., 1992). The concentration of inhibin B in the early follicular phase declines with age, suggesting that it may reflect the follicular reserve. In our women with radiation induced ovarian failure inhibin B was undetectable, as would be predicted. However in the patient with preserved ovarian function, inhibin B was also undetectable in the early follicular phase of an apparently ovulatory cycle. Our findings are consistent with the suggestion that inhibin B may be a valuable measure

of the follicular reserve in women with iatrogenic, and possibly also pathological or physiological, incipient ovarian failure (Klein et al., 1996).

The basis for poor uterine function following radiotherapy is unknown. Uterine blood supply, endometrial function and uterine distensibility have all been implicated (Hawkins et al., 1989). In the present study, women with ovarian failure after TBI showed an increase in uterine volume, blood supply and endometrial thickness after 3 cycles of pSSR. A previous study (Critchley et al., 1992) employing this regimen of pSSR did not demonstrate any significant change in uterine characteristics in women irradiated in childhood. There are a number of important differences between this study and the present study. The patients in the study by Critchley et al were treated at a younger age, with whole abdominal irradiation, with 20 - 30 Gy and they were assessed during only one cycle of pSSR. It may be that further exposure to pSSR would improve uterine characteristics. Wallace et al (1989b) reported poor uterine function for women who had preserved ovarian function following whole abdominal irradiation, with all pregnancies ending in mid trimester miscarriage. For the women treated with TBI it is uncertain whether there would be a further improvement in uterine characteristics with longer exposure to pSSR than the 3 months investigated here. Assessment of uterine distensibility was not undertaken in this study, but may provide further information as to degree of damage to the uterus and the potential to support a pregnancy.

We have shown that uterine volume increased with exposure to pSSR although remained significantly smaller than the comparison group. There was a significant

positive correlation between uterine volume when exposed to pSSR and age at irradiation. The younger the women at TBI, the smaller the uterus which might explain the report of Sanders et al., (1996) that all pregnancies among females who received TBI while pre pubertal resulted in spontaneous miscarriage. Pre pubertal treatment with TBI appears to increase the chance of preservation of ovarian function but the prospect of a successful pregnancy outcome for these women is more uncertain. Women treated post pubertally were noted to have a larger uterine volume and viable pregnancies have been reported for this group. However there is an increased risk of premature labour and low birth weight which is not seen in girls treated with myelo-ablative doses of chemotherapy (Sanders et al., 1996).

Uterine blood flow may be assessed by the uterine artery pulsatility index which varies through the menstrual cycle (with times of greatest impedance to flow during menstruation, and least impedance at times of possible implantation (Steer et al., 1990)). In the absence of SSR, uterine artery blood flow was undetectable in 3 of the 4 women treated with TBI, but with pSSR the pulsatility index was observed to be within the normal range. The uterine artery therefore remains responsive to sex steroids following irradiation but it is not clear whether this observed improvement in uterine blood flow will be sufficient to sustain a successful pregnancy.

Appropriate endometrial development is crucial to the success of implantation and placentation. The endometrium fails to respond to sex steroid replacement only in exceptional cases, for example following pelvic radiotherapy (Critchley et al., 1993; Li et al., 1991; Sauer et al., 1997). With the current regimen there was a correlation

between endometrial thickness and urine oestrone concentration. Therefore optimum levels of serum oestradiol should be achieved for maximal endometrial response. This may be achieved with serial plasma monitoring and increasing transdermal oestradiol replacement. The thickness may also correlate with age at treatment, those with smaller uterine volume treated at a younger age may have reduced thickness. Our numbers were insufficient to investigate this relation. Embryo transfer in certain in vitro fertilisation centres is considered when endometrial thickness is > 6 mm. We have not speculated on thickness that would be suitable for IVF, as we do not feel this is the only item to be considered. Uterine size is also likely to be a limiting step to successful IVF for these women. In our study we obtained an endometrial sample from a patient who had been treated with TBI post puberty. This demonstrated normal luteal phase histology (Noyes et al., 1950) and a similar expression of oestrogen and progesterone receptors to that found in normal tissue (Snijders et al., 1992; Critchley et al., 1993; Critchley at al., 1998).

In considering donor oocytes for such women with ovarian failure, uterine function will be important. The uterine response to physiological sex steroid serum concentrations may be an encouraging guide, on an individual basis, if whether it is sensible to pursue assisted reproduction with donor oocytes as a method of achieving fertility. The validity of such measures as prognostic tests remains to be prospectively demonstrated.

In conclusion, premature ovarian failure after TBI is common and risk relates to age at treatment. Further, these data indicate that a physiological regimen of sex steroid

replacement will improve uterine characteristics. These are encouraging observations for a group of young women who may in the future be candidates for assisted reproductive technologies. Uncertainty however still remains about the most appropriate dose and delivery route for hormone replacement among survivors of childhood cancer. The ideal regimen should aim to benefit the cardiovascular, skeletal and reproductive health of these young people.

CHAPTER 7: Pubertal induction and cyclical sex steroid replacement in women with premature ovarian failure

7.1 Introduction

7.1.1 Background

The onset of puberty in girls is determined by development of the breast buds and an acceleration in growth velocity. The mean age for onset of puberty is 10.8 years with a normal range from 8 to 13 years. There is a gradual progression in development of secondary sexual characteristics, with onset of menses occurring at a mean age of 12.8 years, with a normal range of 8 to 13 years. Some girls are at significant risk of failure of normal pubertal progress, either as a consequence of gonadal failure (for example, girls with Turner's syndrome, autoimmune ovarian failure or galactossaemia, or following treatment for childhood cancer) or lack of central drive (hypogonadotrophic hypogonadism).

The changes are driven by increasing activity of the hypothalamic pituitary axis, with gradually increasing levels of oestradiol. For girls with ovarian failure before the onset of puberty, pubertal induction is required for development of normal secondary sexual characteristics and for skeletal maturation, to achieve growth acceleration and optimize bone mineral accretion. The aim of induction is to mimic the natural timing and rate of progression, although there may be advantages for some girls in delaying the onset of induction to improve final height. Girls are given gradually increasing doses of an oestrogen. A prerequisite of the choice of preparation is that it is available in a small enough dosage to mimic early oestrogen exposure in normal

puberty. For young women with ovarian failure, who have either progressed through puberty spontaneously or had puberty induced with low dose oestrogen, cyclical hormone replacement therapy, with an oestrogen and a progestin, is required. A progestin enables the endometrial lining to be lost on a cyclical basis, and therefore the risk of endometrial hyperplasia, and the inconvenience of menstrual spotting are significantly reduced.

Induction of puberty has been mainly achieved using oral synthetic oestrogen and progesterone. Although the treatment is effective, it has disadvantages. Oral oestrogens have variable bioavailability due to intestinal and hepatic first pass metabolism, which also affects hepatic activity and the clotting system. The natural form of estrogen in humans is estradiol, but it is relatively ineffective after oral administration because of extensive degradation in first pass metabolism in the liver.

There is no agreed consensus on the optimum oestrogen and progesterone preparations for young women with premature ovarian failure. The aim is to achieve optimal reproductive, skeletal and cardiovascular health, but the evidence is not available to dictate best practice (Conway., 2001).

7.1.2 Skeletal health

Oestrogen is a potent stimulator of bone mineral accretion through puberty and into the third decade, when peak bone mass is achieved (Slemenda et al., 1994). An

adequate peak bone mass reduces the risk of osteoporosis and fractures in later life. Premenopausal levels of oestradiol in women with normal ovarian function protect the female skeleton from rapid demineralisation (Howell et al., 1999). However, bone loss proceeds at an increased rate following the menopause (Krolner et al., 1982). In Turner syndrome evidence suggests that women do not achieve peak bone mass and have a higher rate of fractures (Davies et al., 1995). In girls with Turner syndrome the rate of bone mineral acquisition on the OCP is less than is seen in healthy girls, with a 25% reduction in bone mineral content (BMC) from that predicted for age, height, weight and bone size which may be due to suboptimal oestrogen replacement (Shore et al., 1982). Studies have suggested that prolonged OCP use in skeletally immature females leads to a lower peak bone mass (Register et al., 1997). A decrease in BMC and an increased risk of osteoporosis have been demonstrated in survivors of childhood cancer (Hoorweg-Nijman et al., 1999). This may be due to both suboptimal oestrogen replacement and the treatment received for the cancer. Optimising oestrogen replacement may improve bone mineral accretion and reduce the risk of long term morbidity from osteoporosis in women with ovarian failure.

7.1.3 Cardiovascular health

Optimal sex steroid replacement may reduce the risk of cardiovascular disease in young women with premature ovarian failure. The cardiovascular protective effects of oestrogen are well documented in older women who have had endogenous

protection for many years before the menopause (Mendlesohn et al., 1999). However, The Women's Health Initiative has shown that continued exposure to HRT beyond the normal timing of the menopause increases the risk of cardiovascular events (Manson et al., 2003)

Direct actions of oestrogen on blood vessels and effects on serum lipid concentrations reduce the risk of cardiovascular disease in premenopausal women. The vasculature, like the reproductive tissues, bone, liver and brain, is now recognised as an important target organ of oestrogen's action. Oestrogen increases vasodilatation and inhibits the response of blood vessels to injury and the development of atherosclerosis. The incidence of atherosclerotic disease rises in postmenopausal women. There is no current information regarding cardiovascular risk factors in women with premature ovarian failure treated with current hormone replacement regimens. However women with Turner syndrome have a higher incidence of hypertension and cardiovascular disease. Radiotherapy and chemotherapy have a deleterious effect on cardiovascular function, with recognised risks for decreased cardiac function being radiation exposure and chemotherapy, particularly anthracycline treatment (Hansen et al., 1989, Stewart et al., 1995). Thus these women are at increased risk of cardiovascular disease.

7.1.4 Reproductive health

Current SSR may not achieve optimal reproductive health. Women with Turner syndrome who have achieved pregnancy with donor oocytes have an increased risk of miscarriage, still birth and premature delivery (Kaneko et al., 1990). This may relate to poor endometrial or uterine function (Yaron et al., 1996). Uterine volume in women with Turner syndrome who have required pubertal induction with exogenous steroids is reduced and this may effect their reproductive potential (Paterson et al., 2002). Treatment for childhood cancer may have major effects of uterine function if the uterus lies in the radiation field (Hawkins et al., 1989; Critchley et al., 1992). Changes in uterine size and blood flow may be used as an internal control, as changes in uterine parameters are a biological marker of response to SSR. Physiological SSR improves parameters of uterine function, which is a valuable marker of response in a biological target organ (Bath et el., 1999).

We evaluated the current practice within the UK to determine the most commonly used regimens. The aim of the study was to provide evidence of current practice, so that future studies evaluating physiological regimens could be compared to current UK practice.

7.2 Methods

The prescribing habits of all members of European Society of Paediatric Endocrinology practicing in the United Kingdom were surveyed. A questionnaire was sent enquiring as to their choices of hormone replacement for pubertal induction and subsequent hormone replacement in young women with premature ovarian failure. There was an option to document first, second and third choice of HRT. The respondents were asked the rationale for their first choice of HRT. The questionnaire was not anonymised. Those who had not replied within the first 6 weeks were sent a reminder.

7.3 Results

Forty two questionnaires were sent, and the response rate was 71% (n = 30). All respondents, except one, used oral ethinyloestradiol, available as 2 and 10 microgram tablets, for pubertal induction. Hormone replacement choice for the post pubertal girl produced a much more varied response as shown in table 7.1. Two respondents did not have a preferred hormone replacement regimen.

The only consistent reason given for choice of oral contraceptive was convenience and patient's acceptability.

Table 7.1. Choice of hormone replacement for post pubertal women with premature ovarian failure (n = 28)

Method of hormone replacement	n	(%)	Preferred preparation in each category	n	(%)
Combined oral contraceptive	18	(64)	Loestrin (20 or 30)	12	(67)
Hormone replacement oral (cyclical progestin)	5	(18)	Prempak C	5	(100)
Hormone replacement transdermal (cyclical progestin)	3	(11)	Estracombi	2	(66)
Ethinyloestradiol (cyclical progestin)	2	(7)	Ethinyloestradiol (cyclical progestin)	2	(100)

7.4 Discussion.

There was consensus regarding the preparation used to induce puberty. However, there is evidence that use of a more physiological regimen, with transdermal oestrogen, increasing slowly over 4 – 5 years, with the dose tailored to an individuals levels, may have benefits on uterine growth (Piippo et al., 2004). There was no negative effect on final height, and benefits of cardiovascular and skeletal health were not demonstrated in these small studies.

There was no consensus in prescribing cyclical HRT for young women with ovarian failure who face many decades of oestrogen replacement therapy. The reason given for the choice of oral contraceptive pill was convenience of preparation, ease of use and social acceptability. Although this is a justifiable rational for prescribing, it may not be the optimal preparation for skeletal and cardiovascular health.

Further research is required to determine the optimum sex steroid replacement regimen for women with premature ovarian failure. The aim of treatment should be to achieve optimum psychological and physical well being. The long term follow up of women with premature ovarian failure needs to be within a multidisciplinary team who can also address the varying health aspects of both the underlying cause for the premature ovarian failure, and the need to optimise future health.

CHAPTER 8: Spontaneous conception in a teenager who had ovarian cortical tissue cryopreserved before chemotherapy and radiotherapy for a Ewing's sarcoma of the pelvis

8.1 Case report

A 14.9 year old girl who presented with a 2 year history of left groin pain. Radiological investigation demonstrated a bony mass, 110mls in volume, arising from left superior pubic ramus that, on biopsy, was confirmed to be a Ewing's sarcoma. There was thought to be a high risk of ovarian failure resulting from the proposed radiotherapy to the tumour site. After detailed discussion informed consent was obtained from the girl and her parents for laparoscopic collection of ovarian cortical strips. These were stored in Leibovitz medium at –176 °C. Treatment was commenced in accordance with the EICESS 1992 protocol; 14 courses of chemotherapy, with a total dose of ifosfamide of 86.4g/m^2, and 55 Gray to her left pelvis. The radiation field was as shown in figure 8.1. The treatment was completed aged 15.8 years.

Ovarian function was monitored following diagnosis. After completion of radiotherapy aged 15.3 years, there were symptoms and biochemical evidence of ovarian failure; hot flushes, elevated gonadotrophins and undetectable inhibin B (see figure 8.2). Loestrin 20 was commenced and continued until completion of chemotherapy. There was intermittent vaginal spotting and HRT was discontinued for three months on completion of treatment. Re-evaluation of her biochemical data confirmed ovarian failure with significant elevation of gonadotrophins and undetectable inhibin B with absent menses. HRT was restarted but persistent vaginal bleeding continued. A variety of preparations were tried, as detailed in figure 8.2.

Figure 8.1 MRI scan of pelvis for radiotherapy planning – numbers indicate percentage of total dose received by each area

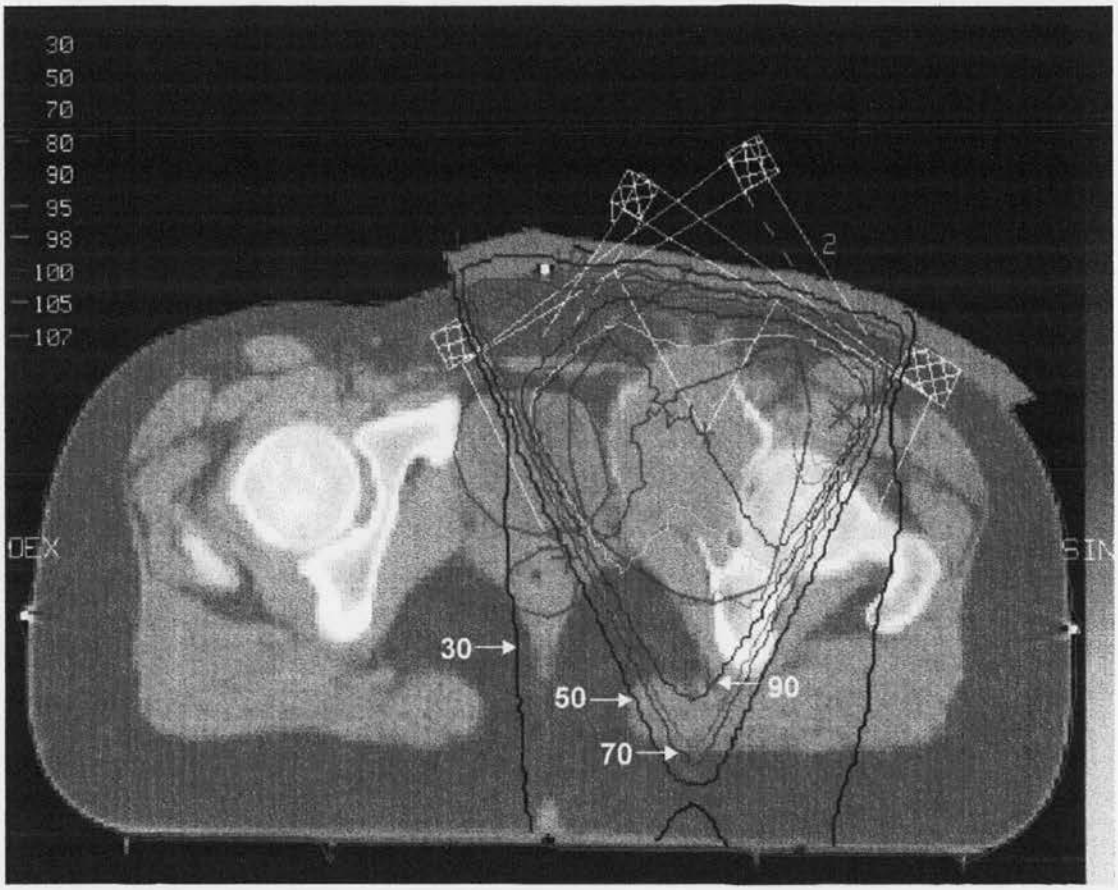
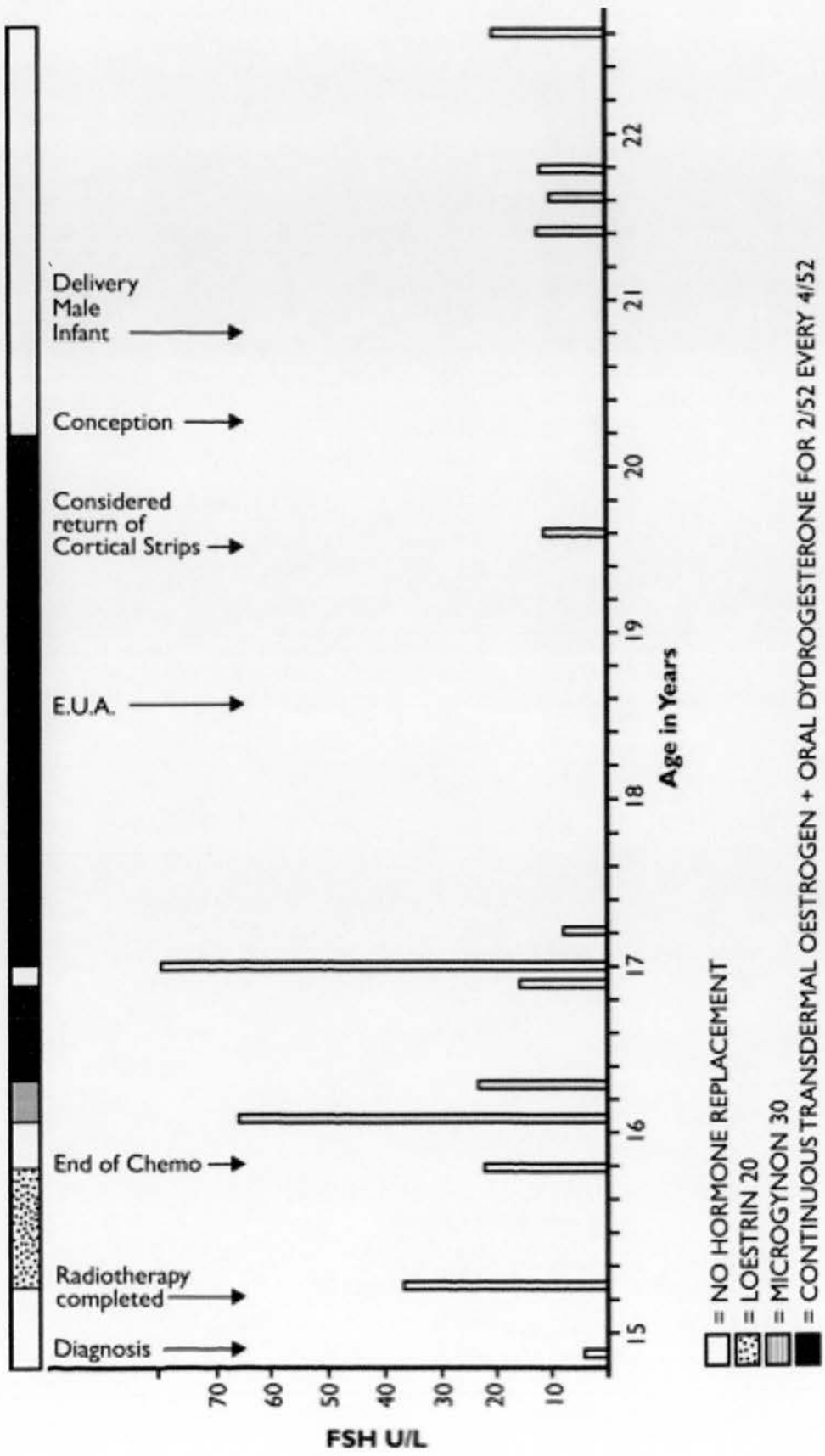


Figure 8.2 Time line indicating events, blood results and HRT.



Aged 16.7 years, while on HRT, a transabdominal ultrasound scan of her pelvis demonstrated a uterine volume of length of 5.9 cm (AP 2.6 cm, transverse 3 cm) with an endometrial echo. The left ovarian volume was 2.2 mls and the right 4.2 mls. No evidence of follicular activity was seen and no vascular signals were obtained from either ovary. Hormone replacement therapy was discontinued for 3 weeks after which gonadotrophin levels indicated ovarian failure (see figure 8.2). Continuous transdermal oestradiol with oral progesterone every 2 weeks was then recommenced. Further evaluation of the persistent vaginal bleeding, on the above regimen, at the age of 18.5 years included an examination under anaesthesia, a cervical smear, a biopsy of vaginal wall, a hysteroscopy and a pipelle biopsy. Examination revealed an atrophic lower vaginal tract. Biopsy of the vaginal wall showed chronic inflammation and ulceration thought to be a radiation effect. The uterine cavity appeared normal and the pipelle biopsy showed normal early secretory phase endometrium. Radiation damage to the lower vaginal tract was thought to be the cause for the bleeding and topical oestrogen was prescribed in addition to transdermal oestradiol. The intermittent spotting improved.

The question of fertility was raised at the age of 19.7 years following the onset of sexual activity and decision to get engaged. There was no dyspareunia or vaginal bleeding. Consideration was given to reimplantation of cortical slices and consultation was organised with the Gynaecology Department. After discussion the decision for reimplantation was deferred for personal reasons and hormone replacement therapy continued.

An ultrasound scan was organised aged 20.2 years following a history of abdominal pain and vaginal bleeding. The USS demonstrated an early intrauterine pregnancy. The bleeding resolved and the pregnancy progressed. Maternal and fetal health were monitored throughout the pregnancy. Cardiac assessment was necessary as the chemotherapy protocol included cardiotoxic agents (anthracyclines). Pelvic assessment was required to consider optimum mode of delivery in view of previous pelvic mass and radiotherapy. Regular fetal growth assessments were performed in view of concern of potential effect of radiotherapy on uterine function. Fetal growth progressed normally.

The history of radiotherapy and pelvic mass indicated that vaginal delivery might be difficult. There was the risk that operative complications may have been avoided by normal delivery. If normal delivery had not been possible then emergency caesarian section could have been a technically difficult procedure. After discussion between colleagues and with the family a decision was made to proceed to elective caesarian section. At 38 weeks gestation a healthy baby boy was delivered weighing 2940g (3^{rd} – 10^{th} centile). Intra operative examination of the pelvic structures revealed normal ovaries and pelvic bones with no evidence of radiation damage.

After delivery there was spontaneous return of menses. Menses have remained regular, occurring approximately once every 28 days without any hormone replacement therapy. Early follicular gonadotrophin levels have been within or just above the normal reference range (see figure 8.2). A pelvic ultra sound scan 15 months after delivery showed uterine length to be 7.3 cm, (AP 3.7 cm, transverse

5.3cm). The uterine artery blood flow was normal. The left ovary was not seen. The right ovary was unremarkable and measured 1.9 x 1.8 x 1.5 cm (volume 2.3 mls).

8.2 Discussion

The treatment for pelvic Ewing' sarcoma is thought to carry a significant risk of ovarian failure. In this case, there was clinical and biochemical evidence of ovarian failure on completion of treatment with elevated FSH levels and undetectable inhibin B. The left ovary received the full dose of radiation (55 Gy), but it is harder to estimate the dose received by the right ovary. The position of the ovary varies, dependent on bladder and bowel contents. The best estimate is that the right ovary received less than 30% of the total dose (< 16.5 Gy); therefore there could have been a significant scatter dose to the right ovary and therefore depletion of primordial follicles (Wallace et al., 2003). The effect of the chemotherapy is hard to determine. There are no good data regarding gonadal toxic doses of ifosfamide. Cyclophosphamide, an analogue of ifosfamide, is associated with gonadal toxicity. Doses is excess of 200mg/m^2 are associated with ovarian failure (Sanders et al., 1991). The ovarian failure is likely to be a consequence of both radiotherapy and chemotherapy. After delivery there was spontaneous return of menses, with gonadotrophins within the normal range. However at 15 months post delivery there was evidence of incipient ovarian failure with elevated FSH and small ovarian volume consistent with a decreased primordial follicle pool, as a consequence of the previous therapy.

The risk of ovarian failure can be estimated prior to gonadal toxic therapy and information regarding the risk should be discussed with the patient. Exact prediction

is not possible as this case demonstrates, but fertility preservation should be discussed with those deemed at high risk of ovarian failure. A number of strategies to protect the ovaries and preserve fertility during cancer therapy have been attempted with limited success. Limitation of radiation dose to the ovary is sometimes practiced in adult women but in children is technically difficult. Ovarian translocation will reduce the radiotherapy exposure to the gonad (Leporrier et al., 1987). Gonadotrophin suppression has been shown to offer some protection from chemotherapy induced ovarian damage (Blumenfeld et al., 1996) but no benefit has been shown in radiation induced ovarian failure. For prepubertal girls, and the majority of young women, preservation of fertility remains experimental and harvesting and storage of gonadal tissue before commencing cancer therapy is the most promising option (Grundy et al., 2001a; Grundy et al., 2001b; Multidisciplinary Working Group, 2003; Poirot et al., 2002). There are reports in the literature of return of ovarian function following reimplantation of cortical strips in ovariectomised sheep and subsequent conception (Gosden et al., 1994). Reimplantation and return of ovarian activity has been reported in a woman who had received gonadal toxic therapy prior to ovarian cortical collection, but no live births reported (Radford et al., 2001).

Ovarian function following gonadal toxic therapy can be evaluated clinically, biochemically and radiologically. Ovarian failure presents with absent menses and symptoms of oestrogen withdrawal, elevated gonadotrophins and small ovaries. Incipient ovarian failure may be detected by early follicular biochemical assessments of gonadotrophins and inhibin B (Creus et al., 2000). Recently anti-Mullerian

hormone has been shown to be a potential marker of ovarian reserve (de Vet et al., 2002; Bath et al., 2003). Radiological assessment of ovarian volume may also be a potential predictor of reserve, with ovarian volume correlating with number of remaining follicles (Syrop et al., 1999; Larsen et al., 2003).

Return of ovarian function and conception years after gonadal toxic chemotherapy and biochemical evidence of ovarian failure has been previously reported (Nasir et al., 1997). There are few reports of return of ovarian function after radiation induced ovarian failure (Chao et al., 1998). Destruction of primordial follicles results in decreased ovarian reserve. Early ovarian failure following therapy has been related to lack of maturing antral follicles due to lack of follicle recruitment and atresia of antral follicles during chemotherapy. Early data from autopsy specimens of children with leukaemia directly demonstrated a reduction in antral follicle number following chemotherapy (Himmelstein-Braw et al., 1978). Return of ovarian activity several months after completion of therapy and after documented biochemical ovarian failure is well recognised. The reason for return of ovarian activity years after documented evidence of ovarian failure is not known. Women with premature ovarian failure after gonadal toxic therapy should be aware of the small but possible chance of return of function and therefore conception.

Chemotherapy has not been shown to have any deleterious effect on uterine function in contrast to pelvic irradiation (Critchley et al., 1992). Radiotherapy to the uterus increases the risk of miscarriage with a high risk of mid trimester loss in women treated prepubertally with pelvic irradiation (20-30 Gray)(Wallace et al., 1989). The

risk relates to age at irradiation (Bath et al., 1999). Pre pubertal radiation exposure increases the risk of mid trimester losses (Sanders et al., 1996).

Collection of ovarian tissue as a potential method of preserving reproductive potential is available in certain centres. In this case, consideration had been given to reimplantation of cortical strips as fertility was not thought possible, given the clinical and biochemical evidence of ovarian failure. The spontaneous conception could have then been attributed to successful re-engraftment and function of the stored cortical tissue. Currently there are no reports of conception post reimplantation of ovarian cortical strips in humans. Autologous ovarian transplantation carries future risks of recurrence of the original cancer and we believe that in vitro maturation of ovarian follicles that have survived freeze/thawing is likely to be available in the future with less risk. The cryopreservation of ovarian cortical tissue is an experimental method of preserving primordial follicles and, with the development of in vitro maturation, may have the potential to preserve fertility in young women treated for cancer.

CHAPTER 9: Conclusions

9.1 Introduction

9.1.1 Background

Fertility is a major concern for women who have survived cancer during childhood, and is of increasing importance as currently 70% of children treated for malignant disease will become long term survivors. The work in this thesis has further informed the issues related to hypothalamic ovarian function after cranial irradiation, investigation of ovarian reserve in women potentially at risk of premature menopause, the risk of ovarian failure post TBI, the uterine characteristics post TBI, and the current practice regarding HRT in those women with ovarian failure.

Women with ovarian failure need an evidence base to aid choice in hormone replacement. For those girls who require potentially sterilising therapy, options for fertility preservation should now be considered. For those women who have had non-sterilising therapy, they remain at risk of a premature menopause and ongoing review of this population is imperative to document evolving late effects.

9.1.2 Ovarian function after low dose cranial irradiation.

The data demonstrate that low dose cranial irradiation has an adverse effect on the hypothalamic – pituitary – ovarian axis that may be progressive over time. The

women had regular menses and normal ovulatory cycles, evidenced by an LH surge and rise in P3G. Early follicular gonadotrophin and inhibin B levels were normal. Ultrasonography demonstrated normal ovaries. These indices are reassuring regarding current ovarian reserve.

However, subtle effects on hypothalamic – ovarian function were demonstrated. LH secretion was decreased throughout the cycle, especially during the LH surge. Short luteal phases were demonstrated; the LH surge was most deficient in those cycles with short luteal phases. Increasing time since treatment increased the chance of a poor LH surge. The function of the corpus luteum is dependent on LH secretion, especially the magnitude and duration of the LH surge. Oestrone secretion was lower during the follicular phase and the luteal phase. Oestrone production is under the influence of both LH and FSH, and decreased LH secretion was demonstrated throughout the cycle.

Apparently minor disturbances in LH secretion may have an effect on reproductive potential: conception in normal women is more likely in cycles with greater LH surges and higher luteal phase progesterone and oestradiol. Short luteal phases are associated with reduced fertility and early miscarriage.

Detailed analysis of reproductive endocrine function at long term follow up of treatment for childhood ALL has not been previously reported. Other reports include a variety of primary diagnoses and therefore varying treatment strategies. Therefore direct comparison is not possible.

Survival after treatment for childhood ALL is greater than 70%, and current protocols aim to increase the survival rate while reducing treatment induced late effects. Low dose cranial irradiation is no longer standard therapy, following the recognition of significant late effects and the confirmation that alternative CNS directed therapies are as effective. There remain a significant number of survivors of leukaemia who have received treatment with chemotherapy and cranial irradiation. The data regarding long term fertility is encouraging. Continued assessment of these women will provide further information regarding fertility rates, and detailed evaluation will provide information on both hypothalamic and ovarian effects over time.

9.1.3 Depletion of ovarian reserve after treatment for cancer in childhood.

The agents used to treat the childhood malignancies will potentially destroy a significant number of the finite number of follicles. The number cannot accurately be predicted, even if the chemotherapy doses and radiation schedules are known. It would therefore be of great value to be able to assess ovarian reserve in women with regular menses or on the OCP.

The data demonstrated that in women with regular cycles, serum FSH was significantly higher and serum AMH significantly lower in the cancer survivors

compared with controls. Other markers of ovarian function, inhibin A, B and oestradiol were not significantly different from controls. Ovarian volume, but not antral follicle count, were also reduced in survivors compared to controls. These data are consistent with cancer survivors having a near normal compliment of small antral follicles. AMH is produced by the granulosa cells of small growing follicles and may therefore reflect more accurately ovarian reserve. AFC and inhibin B reflect the number of FSH sensitive small antral follicles. The higher FSH levels in the survivors may drive increased follicle recruitment, and hence normal antral follicle count and inhibin B levels.

During COCP administration inhibin B was suppressed to undetectable levels in both cancer survivors and controls. In response to FSH stimulation, all controls showed a response, whereas in the cancer survivors, only 6 of 10 showed a response. The AFC was significantly lower in the cancer survivors compared to controls. The results suggest that cancer survivors show a degree of depletion of ovarian reserve and under conditions of hypogonadism, this is reflected in a reduction in the number of FSH sensitive antral follicles.

The underlying diagnoses of the cancer survivors were varied and therefore no comment can be made on individual treatment regimens and the related risk of depletion of ovarian reserve. However, all these women had received non-sterilising chemotherapy in childhood, and these results indicate that there is an effect on ovarian reserve. Detailed follow up of these women is therefore essential to clinically confirm these findings.

9.1.4 Ovarian and Uterine characteristics after TBI

Following TBI in childhood and adolescence we have confirmed that the majority of women have permanent ovarian failure. The risk is greater for those treated post pubertally, but even those with preserved ovarian function have evidence of reduced ovarian reserve.

At baseline, all women with ovarian failure had a small uterus, with poor blood flow and absent endometrium. Following physiological sex steroid replacement for 3 months all measures of uterine function improved such that there was no significant difference in uterine blood flow and endometrial thickness from the comparison group. Uterine volume, although increased with pSSR, remained significantly less than the comparison group. There was a correlation between age at irradiation and uterine volume, indicating that those treated prepubertally had smaller volumes. Optimising hormone replacement may therefore improve uterine function, particularly uterine blood flow and endometrial thickness. However, caution should remain regarding uterine distensibility, especially in those treated prepubertally, as uterine volume remained significantly smaller and there are several reports in the literature of 2nd trimester miscarriage in this group of survivors.

9.1.5 Choice of HRT in females with premature ovarian failure

There is no consensus regarding the choice of hormone replacement therapy in females with premature ovarian failure. The evidence is not available to dictate best practice in terms of skeletal, cardiovascular, reproductive health or psychological well being.

9.2 The future

The treatment of childhood cancer carries a risk of depletion of ovarian follicles. We have demonstrated a high risk with TBI, but a low risk with standard treatment for ALL. Treatment regimens aim to cure but the identification of therapies that confer a significant risk of long term damage has driven changes in chemotherapy regimens, for example as in the treatment of Hodgkin's disease. However, for certain malignancies, the risk of ovarian damage has been accepted given the increased chance of a cure. Identification of which therapies are associated with a high risk of ovarian failure has therefore been a driving force in research into preservation of ovarian function for the prepubertal girl.

Studies have evaluated suppression of the hypothalamic pituitary ovarian axis with gonadotrophin releasing hormone analogues in post pubertal women. There has been no evidence that this achieves significant gonadal protection and does not seems to halt the decline in numbers of primordial follicles.

The options for preserving fertility for the prepubertal girl are limited. There has been much interest in the cryopreservation of primordial follicles for young girls as a method of preserving reproductive potential. In the 1950s autografts of mouse ovarian tissue were shown to survive after freezing and thawing (Parkes., 1957). Normal offspring were obtained from mice with orthotopic ovarian grafts that had been frozen and stored, although the reproductive life of the females was short

(Parrot., 1960). In the 1990s this method of restoring fertility to oophorectomised animals was studied in sheep (Gosden et al., 1994). Ovarian cortical strips were frozen and reimplanted after oophorectomy. Return of oestrous cycles was demonstrated although gonadotrophin levels remained higher than the precastrate range.

The collection and storage of ovarian cortical tissue for girls and young women before gonadal toxic therapies has been a possibility since the mid 1990s, and has been available in some centres. Tissue is collected laparoscopically under general anaesthesia. The tissue is then stored in cryoprotectant and frozen at –196°C in liquid nitrogen. The Royal College of Obstetricians and Gynaecologists has produced a report from a working party on the storage of ovarian and prepubertal testicular tissue (RCOG Storage of ovarian and prepubertal Testicular tissue., 2000) . This provides standards for best practice in cryopreservation of gonadal tissue, including the criteria for providing a service, patient identification and selection, standard operating procedures and aspects of storage.

The future use of the tissue will be dictated by available options. Currently the only method by which follicle maturation is possible is reimplantation of the tissue, onto structures within the abdominal/pelvic cavity. The functional lifespan of the strips is related to the number of follicles that survive the re-engraftment process, estimated to be approximately 70% (Oktay et al., 1997). One of the advantages of ovarian cortical tissue is that there are many primordial follicles potentially able to survive the freeze thaw process. The younger the child, the greater the number of primordial

follicles present in the harvested tissue. There are concerns that the reimplanted tissue may transmit the original disease, as the gonads are recognised sanctuary sites for malignant cells, particularly for haematological malignancy (Shaw et al., 1996; Oktay et al., 2000).

There is debate as to who should be offered the collection of ovarian cortical tissue. There is therefore an urgent need to provide consensus as to the future for fertility preservation in children (Wallace et al., 2001). It is available in some centres and there is increasing public awareness of its potential to preserve fertility. The criteria that have been agreed locally are shown in table 9.1.

Although it has been demonstrated that human primordial follicles survive cryopreservation and return of ovarian hormonal activity has been achieved with reimplantation, it remains an experimental procedure. However, one pregnancy has now been reported and the authors argue that the follicle matured from the transplanted tissue, not the remaining ovarian tissue (Donnez et al., 2004).

For those women with ovarian failure there is the option to consider donor oocytes. However, given our data regarding uterine function post radiation, clinicians should be cautious about the chance of a successful outcome in women in whom the uterus has been exposed to radiotherapy prepubertally.

For those women not at high risk of ovarian failure, we have shown that ovarian reserve can be evaluated with biochemical markers, and ultrasound imaging. A

recent paper (Wallace at al., 2004) evaluated the use of ovarian volume in prediction of ovarian reserve. They demonstrated a highly significant correlation between primordial follicle population and ovarian reserve, and suggested that this would revolutionise the management of women seeking advice regarding reproductive status. However, further longitudinal studies need to be done to monitor reproductive function in women who have had treatment that may deplete ovarian reserve. This would further inform clinicians about the long term risks of treatment and enable clinicians to inform patients about the potential risks.

For those women that have ovarian failure, there is a need to optimise hormone replacement. Evidence is required to dictate best practice. Studies in women with ovarian failure evaluating the benefits of a physiological regimen versus standard replacement therapy are ongoing. The choice of the standard HRT within one arm of the study was dictated by the questionnaire regarding current UK choice of HRT in women with premature ovarian failure. However, the final choice of HRT should be the patients own, once the various options, and associated risks and benefits have been discussed. Hormone replacement therapy came under question after the report for the Women's Health Initiative (Manson et al., 2003). Although this report was not relevant to young women who have premature ovarian failure, it does highlight the need for an evidence base. Long term studies are therefore required and, given that the numbers of women are small, multi-centre collaboration would be essential.

9.3 Future research plans originating from this thesis

The initial drive to research into the late effects of the treatment of childhood cancer came from the recognition of significant morbidity and mortality from the treatment schedules. The current schedules aim to maximise the chance of cure, while recognising the need to balance the risk of late effects. It is therefore essential to continue to monitor survivors of childhood cancer to determine the effect of current protocols on late effects, including fertility. There is very little population based data regarding long term fertility in survivors of childhood cancer. The UKCCSG has a database of all patients and therefore would facilitate tracing of all survivors. We aim to study the fertility in female survivors as a nationwide study, with an initial pilot study in Scotland. We would take forward the work on assessment of ovarian reserve, using ultrasound imaging to determine ovarian volume and antral follicle count, and early follicular biochemical assessment of FSH, LH, inhibin B and AMH. An initial cross sectional study could be the preliminary work necessary for longitudinal studies assessing ovarian function in cancer survivors.

The current data do not allow for accurate prediction of ovarian reserve for an individual. There is significant intercycle variability in all these markers. Serial measurements improve the reliability of these assessments. Longitudinal data detailing progression in these markers over time, in relation to the timing of the menopause, will determine whether they have a place in prediction for an individual. However, the recognition that conceptions occur in women who have had

documented ovarian failure highlights the fact that even with accurate biochemical and ultrasound assessment it may never be possible to predict with certainty for an individual.

The second line of research we are pursuing is the assessment of optimum hormone replacement in women with premature ovarian failure. Current HRT choice is not evidenced based for the young woman with premature ovarian failure. Although the cohort of survivors with ovarian failure from childhood cancer is small, there are other groups of women who could benefit from optimising HRT. We are currently studying women with Turners syndrome, women with ovarian failure after childhood cancer and women with idiopathic ovarian failure in a crossover study comparing physiological sex steroid replacement regimen against a standard COCP, to determine whether there are benefits as assessed by markers of cardiovascular, skeletal and reproductive health.

Table 9.1. Edinburgh criteria for selection of patients for cryopreservation of ovarian cortical tissue

Age ≤ 30 years
No previous chemotherapy/radiotherapy (if aged <15 years consider if previous 'low-risk' chemotherapy)
A realistic chance of long-term survival
A high risk of treatment-induced immediate ovarian failure (estimated at >50%)
Informed consent (from patient or in the case of an incompetent child from the parents)
Negative HIV and Hepatitis serology
No existing children

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